University of Alberta

Biomonitoring of Tamoxifen and Its Metabolites in Breast Cancer Patients using Nonaqueous Capillary Electrophoresis with Electrospray Mass Spectrometry

by

Spencer J. Carter ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Biomonitoring of Tamoxifen and Its Metabolites in Breast Cancer Patients using Nonaqueous Capillary Electrophoresis with Electrospray Mass Spectrometry** submitted by **Spencer J. Carter** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**.

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Abstract

Tamoxifen is a selective estrogen receptor modulator (SERM) used to treat and reduce the risk of breast cancer. Tamoxifen and several of its metabolites in urine and serum from breast cancer patients were extracted and analyzed. Analysis of these metabolites was performed by nonaqueous capillary electrophoresis with electrospray mass spectrometry (CE/ESI/MS). Peak heights from extracted ion current electropherograms of the metabolites were used to establish a metabolic profile for each patient.

One of the major objectives of this thesis was to develop a method for the precise and accurate analysis of tamoxifen and its metabolites in breast cancer patients using CE/ESI/MS. Separation (CE) and detection (ESI/MS) conditions were optimized. The method was validated with respect to within-day and between-day precision. Calibration curves were constructed from the analysis of standards and absolute concentrations were determined in a few patients.

The following are some of the major findings for the analysis of patient samples: In urine, there were statistically significant differences between the amount of tamoxifen N-oxide found in early stage (stages I, II, and III) compared to advanced stage (stage IV) breast cancer patients. Also in urine, for patients with metastatic breast cancer only, there were statistically significant differences in the amount of 3,4–dihydroxytamoxifen found in those patients continuing to benefit from tamoxifen treatment compared to patients whose cancer had progressed. In serum samples, a statistically significant relationship was demonstrated between the amounts of tamoxifen and breast cancer stage (early stage vs. advanced stage). An identical relationship was also identified for the major metabolite in serum, N–desmethyltamoxifen. The most probable explanation for these observations is that the presence of clinically detectable breast cancer deposits influence tamoxifen metabolism. One mechanism for this is intratumoral metabolism of tamoxifen.

Two in vitro tamoxifen experiments were performed. In the first, tamoxifen and its acid hydrolysis products were separated and tentatively identified by nonaqueous CE with thermo–optical absorbance CE/ESI/MS. In the second experiment, the transformation of tamoxifen at 37 °C in synthetic gastric fluid was analyzed by high performance liquid chromatography with triple quadrupole mass spectrometry.

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List of Abbreviations and Symbols

AC	alternating current
AF-1	activation function 1; region of the estrogen receptor
AF-2	activation function 1; region of the estrogen receptor
amu	atomic mass unit
BRCA1	breast cancer gene 1
BRCA2	breast cancer gene 2
°C	degrees Centigrade
C	analyte concentration
^{13}C NMR	carbon-13 nuclear magnetic resonance
CE	capillary electrophoresis
CEM	channel electron multiplier
CID	collision induced dissociated
cps	counts per second
CUR	curtain gas flow rate
CYP	cytochrome P-450
D	diffusion coefficient
DC	direct current
DF	deflector plate
DNA	deoxyribonucleic acid
ε	dielectric constant of the solvent
ε_o	permittivity of free space
E	cis
ER	estrogen receptor
ER-α	estrogen receptor alpha
ER-β	estrogen receptor beta

ERE	estrogen receptor element
ESI	electrospray ionization
GC	gas chromatography
^{1}H NMR	proton nuclear magnetic resonance
HPLC	high-performance liquid chromatography
I	ionic strength
i.d.	inner diameter
ICI	Imperial Chemical Industries
Int. Std.	internal standard
IQ1	Interquad lens 1
IR	infrared
kD	kilodalton
L	capillary length
LBD	ligand binding domain
LC	liquid chromatography
[M + H]	molecular ion and hydrogen
M.W.	molecular weight
m/z	mass to charge
mAU	milli arithmatic units
MEKC	micellar electrokinetic chromatography
MS	mass spectrometry or mass spectrometer
ms	milliseconds
MS/MS	tandem mass spectrometry or tandem mass spectrometer
N	number of theoretical plates
η	viscosity of the solvent
n	frequency
n/a	not applicable

NADPH	nicotinamide adenine dinucleotide phosphate
NEB	nebulizer gas flow rate
NSABP	National Surgical Adjuvant Breast and Bowel Project
o.d.	outer diameter
ODS	octadecyl silane
OR	orifice plate
p	statistical probability
pH	negative logarithm of the hydrogen ion concentration
Pk Ht	peak height
ppm	parts per million
q	charge on the ion
Q	quantity injected
Q0	preliminary quadrupole
Q1	first quadrupole mass analyzer filter
r	radius of the ion
R	resolution
R	correlation coefficient
R^2	correlation coefficient squared
RF	radio frequency
RNG	focusing ring
RSD	relative standard deviation
s/p	serum/plasma
SDS	sodium dodecyl sulfate
SERM	selected estrogen receptor modulator
S/N	signal to noise
ST	stubbies
t	injection time

tam	tamoxifen
TGF-β	transforming growth factor beta
TIC	total ion current
t_{inj}	injection time
TLC	thin-layer chromatography
t_m	migration time
TOAD	thermo–optical absorbance detection
t_r	retention time
μ_{eo}	electroosmotic mobility
μ_{ep}	electrophoretic mobility
U.S. F.D.A.	United States Food and Drug Administration
UV	ultra violet
V	applied voltage
V_{inj}	injection voltage
V_m	running voltage
Vol_{cap}	volume of the capillary
Vol_{inj}	volume injected
w	full width of a peak
XIC	extracted ion current
Z	trans
ζ	zeta potential

Chapter 1

Introduction

1.1 Breast Cancer and Tamoxifen

1.1.1 Breast Cancer

One out of every nine women will develop breast cancer in her lifetime and approximately one-third of those will die from it (1). Breast cancer is the most common form of cancer in women. Fortunately, 90% of patients with breast cancer, detected in its early stages, can be cured (2). For those with breast cancer in its later stages, the prognosis is not as good. Those who develop breast cancer will have to make difficult choices concerning surgery and treatment after the surgery. Treatment can include radiation, chemotherapy, hormonal therapy, or a combination of these, although not necessarily simultaneously. Developments have been made with treating breast cancer patients, but much more needs to be done. Drug therapies in breast cancer are somewhat ambiguous. Some patients are effectively treated while others receive no benefits.

1.1.1.1 Risk Factors

Most known risk factors for breast cancer are factors that effect endogenous estrogen levels (3,4). The major risk factors for developing breast cancer are previous breast cancer, age over 50, and a strong family history of breast cancer. About 5% of breast cancers are related to genetic predisposition (5). Mutations in the BRCA1 and BRCA2 genes are implicated in 80% of high-risk families in which three or more members have a history of breast cancer (6-8). These genes, located on chromosomes 17 and 13, respectively, help encode a protein important in the suppression of proliferation of breast cells (8-10). Notwithstanding these risk factors, it should be noted that approximately 75% of women who develop breast cancer have no family history of the disease (11). Minor risk factors include having no children (12) or having a first child after age 30 (13), an onset of menstruation at age 12 or younger (14), and an onset of menopause after age 55 (12). Several unproven but potential risk factors for developing breast cancer also exist such as having a high fat diet (15-17), alcohol intake (18), and exposure to environmental pollutants (19-21).

1.1.1.2 Breast Cancer Stages

Clinical cancer stage is determined by the size of the primary tumor, the presence of chest wall invasion, and the presence or absence of regional or distant metastases. Clinical stages are useful for selecting and evaluating therapy. Cancer stage can be determined by all of the findings of physical examination, imaging studies, operative study, and pathologic examination of the breast or other tissue (22). Table 1.1 outlines the various stages of breast cancer (23).

1.1.2 Tamoxifen

Tamoxifen is a widely accepted treatment for both pre- and post-menopausal patients with hormone-sensitive breast cancer. The structure of tamoxifen is shown in Figure 1.1.

1.1.2.1 History of Tamoxifen

Tamoxifen was first synthesized in 1966 by Imperial Chemical Industries in Great Britain (24). The purpose of the synthesis was to find an agent that could be used for birth control. Tamoxifen was found to be an effective birth control in some animals, but not in mammals (25,26). In the 1960s evidence was found that breast cancer growth depended on hormones, especially estrogen. Proof of this is the shrinkage or remission of breast cancer that occurs with surgical ovariectomy (27). In the early 1970s the first breast cancer trials of tamoxifen began. These trials were primarily for elderly postmenopausal patients. The response rate was similar (20-22%) to other hormonal therapies (such as diethylstilbestrol or aminoglutehimide) at the time (28-30). The exciting result from these trials was that tamoxifen had fewer side effects than these other

Table 1.1. Stages of breast cancer (23).

Stage	Classification*	Extent of Tumor
0	Tis, N0, M0	In situ (confined to lobules or ducts); has not spread beyond membrane of breast
I	T1, N0, M0	Small (greatest dimension < 2 cm); has not spread to lymph nodes in armpit or to other more distant areas of body
IIA	T0, N1, M0 Or T1, N1, M0 Or T2, N0, M0	2 cm or smaller and has spread to lymph nodes in armpit; or 2-5 cm but has not spread to lymph nodes of armpit
IIB	T2, N1, M0 Or T3, N0, M0	2-5 cm and has spread to lymph nodes in armpit, but nodes are not attached to one another; or > 5 cm but has not spread to lymph nodes or other more distant areas of body
IIIA	T0, N2, M0 Or T1, N2, M0 Or T2, N2, M0 Or T3, N1-2, M0	2-5 cm or smaller and has spread to several lymph nodes of armpit, causing node to adhere to one another or to blood vessels; or > 5 cm and has spread to lymph nodes of armpit
IIIB	T4, N0-3, M0 Or T0-4, N3, M0	Any size, but is fixed to chest wall or muscle, or involves lymphatics of skin or skin itself and may or may not involve lymphatic nodes of armpit; or any size but has spread to lymph nodes above collarbone
IV	T0-4, N0-3, M1	Any size; lymph nodes may or may not be involved but cancer has spread to areas other than lymph nodes and breast

**Tissue* (T)

Tis: tumor in situ; T0: no evidence of primary tissue; T1: tumor < 2 cm; T2: tumor 2-5 cm; T3: tumor > 5 cm; T4: tumor any size, accompanied by involvement of chest wall; involvement of lymphatics within skin of breast; infiltration of skin by malignant cells; skin ulceration.

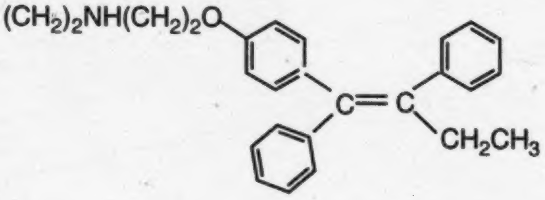
Lymph Nodes (N)

N0: no cancer present in lymph nodes; N1: cancer involvement but nodes are movable; N2: nodes attached to one another or to adjacent blood vessels; N3: cancer spread to nodes above collarbone.

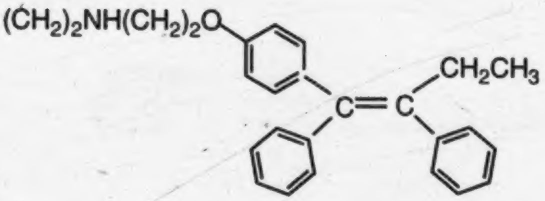
Metastasis (M)

M0: no metastasis; M1: metastasis to a distant site.

Figure 1.1. Structure of tamoxifen isomers.


(CH2)2NH(CH2)2O
C=C
CH2CH3

(Z)–tamoxifen


(CH2)2NH(CH2)2O
CH2CH3
C=C

(E)-tamoxifen

therapies. In addition, tamoxifen was a new alternative for women in their 50s who were within three years of expected menopause (perimenopausal) because this group showed the best responses to tamoxifen and had been previously insensitive to hormone therapy. In 1977 the United States Food and Drug Administration (U.S. F.D.A.) approved tamoxifen for use in postmenopausal women with metastatic breast cancer. Of those patients who used tamoxifen, approximately one-third responded (31). Many of these patients had exhausted other forms of hormone therapy. In 1998, the U.S. F.D.A. approved the use of tamoxifen for the reduction of risk in women determined to be at an increased risk of developing breast cancer. This approval followed the interim finding of the National Surgical Adjuvant Breast and Bowel Project P-1 Study (NSABP-P1) that tamoxifen reduces the risk for developing breast cancer in healthy women at high-risk (32).

1.1.2.2 Prevention of Breast Cancer

The recognition of there being only limited therapies for treatment of breast cancer has resulted in more concentrated efforts to prevent breast cancer (33). In previous years, the only method of prevention available in routine clinical practice was prophylactic mastectomy. Besides the obvious drawbacks of this method, little agreement exists on the efficacy of this procedure (34-36).

In trials of tamoxifen for use as an adjuvant therapy, there was a reduction of approximately 40% in the rate of contralateral breast cancer. This observation was the impetus for trials of tamoxifen used as a preventative measure against breast cancer in healthy women (33).

1.1.2.2.1 Prevention of Breast Cancer through the use of Tamoxifen

The NSABP trial showed that an intake of 20 mg of tamoxifen daily in healthy women who were at high risk for developing breast cancer reduced their risk for developing the disease by 49%. Other potential effects from the intake of tamoxifen were analyzed. Important results are shown in Table 1.2.

The results from the study received widespread media coverage. The results have been controversial. A 49% reduced risk in breast cancer equates to just one out of every 75 women receiving this reduced risk. Two other studies conducted in Italy (37) and Britain (38) reported in 1998 that tamoxifen did not reduce the risk of developing breast cancer in healthy women. Differences in the results of the European and the NSABP studies could be attributed to the fact that these studies looked at women of different ages, used different ways of deciding which women were at risk of breast cancer, and had different follow up duration periods. The Italian group chose women who had had hysterectomies. Women in the British pilot study were older, more likely to have a genetic predisposition to breast cancer, and were monitored for six years or more.

In Canada, tamoxifen cannot be marketed as a drug that reduces the risk of breast cancer, at least not until it is approved for that purpose by the Health Protection Branch. Tamoxifen is available in Canada though as a standard treatment for women who have breast cancer, and that means doctors here can also prescribe it to healthy women.

1.1.2.3 How Tamoxifen Works

How tamoxifen works exactly is not entirely known. What is known is that some types of breast cancer cells contain proteins called estrogen receptors (ER). Estrogen, synthesized in the ovary, can bind to the ER and stimulate cell growth. The major action of tamoxifen is believed to be binding to the ER and thus inhibiting the uptake of estrogen to the ER (39). Other mechanisms for the action of tamoxifen have also been proposed (40).

Table 1.2. Summary of notable results from NSABP-P1 study.

Parameter	**Tamoxifen (n=6576)**	**Placebo (n=6599)**	**Net Change**
Breast Cancer	89	175	49% reduction
Bone Fractures	111	137	19% reduction
Ductal Carcinoma In Situ	31	59	47% reduction
Pulmonary Embolisms	18	6	200% increase
Deep Venous Thrombosis	33	14	59% increase
Endometrial Cancers	33	14	136% increase

Tamoxifen was classified in the past as an antiestrogen or an estrogen antagonist but is known now as a selective estrogen receptor modulator (SERM). An antagonist prevents the action of an agonist by acting on its specific receptor, or site; an agonist is a substance that produces a response (41). The term ‘SERM’ was coined when it was realized that the terms “estrogen agonist”, “estrogen antagonist”, “antiestrogen”, etc. could not account for the actions of some drugs such as tamoxifen and raloxifene (42,43). Raloxifene is used for the prevention of osteoporosis in post-menopausal women (44). The activities of SERMs are tissue selective. SERMs such as tamoxifen and raloxifene produce beneficial estrogen-like effects on bone and lipid metabolism, while antagonizing estrogen in some other tissues. In the uterus, tamoxifen behaves as an agonist, while raloxifene behaves as a complete antagonist. In the breast, tamoxifen has both antagonist and agonist (see Section 1.1.2.5) properties. SERMs are different from full estrogens (e.g. 17β-estradiol or 17α-dihydroequilenin), which behave as agonists in all tissues and pure antiestrogens (e.g. ICI-164384) which behave as estrogen antagonists in all tissue types. The precise mechanism by which SERMs produce this tissue-selective pharmacology remains unknown (45-47).

1.1.2.4 Estrogen Receptor

The estrogen receptor is a member of the nuclear hormone receptor superfamily, a group of structurally related proteins found in nearly all animals. ERs bind to hydrophobic hormones in the cytosol and, following ligand-induced translocation to the nucleus, function as transcription factors. The binding pocket on the ER, which is largely hydrophobic, allows the ER to bind to estrogen, a non-polar ligand, which then activates the ER (48). The binding specificity for ligands to the ER depends on the non-polar interactions and the hydrogen bonding within the ER-ligand complex. The ER can bind to a variety of agonistic and antagonistic ligands because part of the ER's binding pocket is considerably larger than estrogen and is therefore relatively non-specific.

There are two sub-units of ERs, one of them just recently discovered, named ER–β (49-51). The classical ER was renamed to ER–α. Since then, it is becoming apparent that both types of ERs are necessary for normal function, in both males and females, and that ER–α and ER–β have distinct but interrelated functions (42,52,53). ER–β is a 53 kD protein and ER–α is a 66 kD, two-domain protein. In ER–α, the N-terminal is a DNA-binding domain. The C-terminal domain consists of 258 amino acids and is the ligand-binding domain (LBD). The LBD consists roughly of a three-layered fold of anti-parallel alpha helices. The center layer comprises three helices and is sandwiched between two layers of three and four helices. This structure forms a wedge-shaped pocket that allows for binding to ligands like estrogen. In the vicinity of the ligand pocket are a C-terminal helix and a pair of anti-parallel beta-strands (54).

The LBD domain of ER–α was first crystallized in 1997 with estradiol and raloxifene (55). Raloxifene is similar to tamoxifen in that it has antiestrogenic effects in the breast. In these crystal structures, major differences in the structure of the LBD complexes of estradiol and raloxifene were revealed. Estradiol and raloxifene induce two different conformations of helix 12, a major transactivation domain of the ER, resulting in different surfaces of the ER (55). Different ER surfaces suggest that ER will interact differently with coactivators and corepressors, thus partially explaining the different responses of these two ligands (42).

The ER changes its conformation by dimerizing in the LBD upon binding to estrogen. Two coactivators, AF-1 and AF-2, bind to the ER because of this change and they allow the ER to interact with specific DNA sequences more effectively. These specific DNA sequences are known as estrogen response elements (EREs) (56). Interactions with DNA by the ER activate transcription and ultimately cell growth. With pure antiestrogens, the ER is incapable of dimerizing, which may be due to a nonfunctional ligand binding to the LBD, or an increase in ER turnover (57). With SERMs, the activation of gene transcription may depend on the relative ratio of coactivators and corepressors within the cell. With tamoxifen, for instance, the coactivator AF-2 may not be able to bind to the LBD due to the altered shape of the dimerized ER (58). As a result, tamoxifen may interact with DNA, but not to the same extent as estrogen. Coactivators and corepressors appear to act as accelerators or brakes that modulate transcriptional regulation of the hormone responsive target gene expression (59,60).

1.1.2.5 Tamoxifen Resistance

Cells surviving drug therapy are "drug resistant". Two forms of resistance occur. The first is innate resistance where tumor cells are resistant *de novo*, before the drug has been given. This is commonly found in kidney or lung cancer. The second resistance is acquired clinical resistance where cells only become resistant after exposure to the drug. Initially, cancer cells are sensitive to treatment but progressively become less so. This is commonly found in breast cancer, and with tamoxifen, generally occurs about 2-5 years after initial treatment.

Tamoxifen resistance is associated with resistant mechanisms at the level of the breast cancer itself. Many cellular changes have been associated with tamoxifen resistance, but the mechanisms for acquired resistance to tamoxifen remain unknown. Many have proposed mechanisms for this resistance. Some of these proposals include:

- Variability in tamoxifen metabolism among patients and within breast tumors (61,62).
- Estrogenic tamoxifen metabolites contribute to cellular resistance (40,61,63,64).
- A selection process favoring cells that have no ERs or cells that produce mutated ERs, which fail to recognize tamoxifen (65-67).

- Tamoxifen increases the release of TGF-β, a growth-inhibitory factor, from certain cells. Resistance occurs when these cells no longer produce the TGF-β required to continue restraining growth of the breast cancer cells (68,69).
- Tamoxifen binds to the ER, producing a pocket, allowing other proteins to bind to that pocket, which stimulate cell growth (70).
- Selective increase in coactivator molecules (67).

After continued exposure to tamoxifen, tumors can actually become dependant on tamoxifen and can be stimulated by it (71,72). The process for this is not well understood. Studies have shown that in some patients on tamoxifen treatment, whose breast cancer began to grow, discontinuing treatment stabilized or reduced the tumor (73,74).

1.2 Drug Absorption, Distribution, Metabolism, and Excretion

For a drug to be effective, it must reach the target tissue in a high enough concentration to cause a response. This depends on the drug's absorption into, distribution within, metabolism by, and excretion from the body. These events occur simultaneously and the final outcome of a drug is the result of the interaction of all these events (75).

1.2.1 Drug Absorption

A drug must be absorbed through the cell membranes that comprise the various layers of the skin or that line the digestive or respiratory tracts. Only certain substances pass through cell membranes, because they are selectively permeable. Selective permeability for a drug is determined by its molecular size, lipid solubility, and electrical charge, as well as by the active and passive transport mechanisms of the cell membrane (75).

1.2.2 Drug Distribution

Once the drug enters the bloodstream it is transported and distributed by the circulatory system to the various tissues of the body. It is then stored, metabolized, or excreted. Significant absorption into and out of the bloodstream occurs only in the capillaries (75).

1.2.3 Drug Metabolism

Biotransformation, or metabolism, is the sum of all the physical and chemical changes of a drug that take place within an organism. The products of these changes to the parent compound are metabolites. Metabolites can have little or no pharmacological effect (76), be more active than the parent compound (77), or generate additional metabolites, which have responses unrelated to that of the parent compound (78).

Biotransformation includes two types of enzymatic reactions: Phase I, called catabolism (disintegration or tearing down processes), and Phase II, called anabolism (assimilation or building up processes). Catabolism is the breakdown of substances into simpler substances. Anabolism is the conversion of ingested substances into the constituents of protoplasm (79). Phase I reactions include oxidations, reductions, and hydrolyses. Phase II reactions include glucuronidations, sulfations, acetylations, methylations, conjugations with glutathione, and conjugations with amino acids. Phase I and II reactions may occur simultaneously or sequentially. Phase I products are generally less toxic, more water soluble, and easier to excrete. Phase II reactions bind molecules to the parent drug or a metabolite from a Phase I reaction.

The primary organ where these changes take place is the liver. Other changes occur however, in the kidneys, lungs, skin, nasal mucosa, eyes, and gastrointestinal tract. Transformation occurs as a result of the interactions of the drug with an enzyme in the cell's endoplasmic reticulum, cytoplasm, or mitochondria. For most drugs, the enzymes that transform drugs to metabolites are reductase P-450 and cytochrome P-450 in association with nicotinamide adenine dinucleotide phosphate (NADPH). Metabolic reactions affect the efficacy of drugs in disease treatment and determine their pharmacological effects. Therefore, analysis of metabolites from complex body fluids such as urine and serum is necessary for understanding the effects of the parent drug (75).

Metabolism is effected by such factors as age, sex, nutritional status, disease, enzyme induction or inhibition, and genetics. Each of these factors may affect metabolism individually or collectively. For example, babies are more susceptible to chemicals such as pesticides because their cytochrome P-450 enzymes are not well developed. Factors that effect absorption and distribution can also effect metabolism. For example, the absorption rate, binding of the drug to proteins in plasma, and storage will effect the rate at which the drug is delivered to the tissue where metabolism occurs. Generally, organic substances, such as tamoxifen, are easily absorbed, because of high lipid solubility, and therefore undergo metabolism easily.

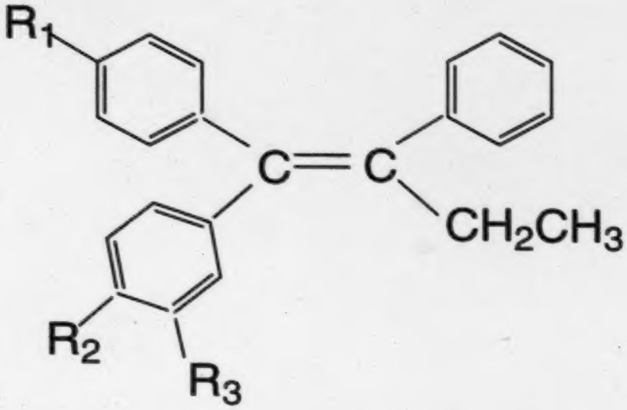
1.2.3.1 Tamoxifen metabolism

Tamoxifen is not unlike other drugs in how the body absorbs, distributes, metabolizes, and excretes it. Tamoxifen produces Phase I and Phase II metabolites in the body. Table 1.3 shows the structures of some of the Phase I metabolites of tamoxifen.

1.2.3.1.1 Isomeric forms of Tamoxifen and Metabolites

Tamoxifen can exist as a trans (Z) or cis (E) isomer (39,80). (Z)–tamoxifen has antiestrogenic properties, whereas (E)–tamoxifen is estrogenic. Tamoxifen that is administered to patients ('Novaldex') contains less than 1% of the cis isomer (39). The structures shown in Table 1.3 are in the Z–isomeric form. The Z and E isomers of tamoxifen are shown in Figure 1.1.

Table 1.3. Structures of tamoxifen metabolites.



Compound	Metabolite	R_1	R_2	R_3	Molar Mass (g mol^{-1})
tamoxifen	-	$(CH_3)_2N(CH_2)_2O$	H	H	371
4-hydroxytamoxifen	B	$(CH_3)_2N(CH_2)_2O$	OH	H	387
4-hydroxytamoxifen-N-desmethyltamoxifen	BX	$CH_3NH(CH_2)_2O$	OH	H	373
3,4-dihydroxytamoxifen	D	$(CH_3)_2N(CH_2)_2O$	OH	OH	403
-	E	OH	H	H	300
N-desmethyltamoxifen	X	$(CH_3)HN(CH_2)_2O$	H	H	357
-	Y	$HO(CH_2)_2O$	H	H	344
N-didesmethyltamoxifen	Z	$H_2N(CH_2)_2O$	H	H	343
tamoxifen-N-oxide	-	$(CH_3)_2NO(CH_2)_2O$	H	H	387

1.2.3.1.2 Pharmacology of Tamoxifen and Metabolites

Some tamoxifen metabolites have an increased affinity for ER–α, such as 4–hydroxytamoxifen and 4–hydroxy–N–desmethyltamoxifen. In fact, 4–hydroxytamoxifen has been reported to be 100 times more potent than tamoxifen in inhibiting tumor growth (81,82). The affinity of 4–hydroxytamoxifen for the ER is at least as great as that for estrogen. Also similar to estrogen, 4–hydroxytamoxifen shows a very slow dissociation rate from the ER, unlike tamoxifen (39,83). Despite these similarities of 4–hydroxytamoxifen to estrogen, studies have shown that the metabolism of tamoxifen to 4–hydroxytamoxifen may be helpful, but not necessary, for the inhibition of estrogen action to occur (39,84). 4–Hydroxytamoxifen has agonistic and antagonistic properties in the breast. 4–Hydroxy–N–desmethyltamoxifen is similar to 4–hydroxytamoxifen in that is has a high affinity for the estrogen receptor and also has agonistic and antagonistic properties in the breast (81). 3,4–Dihydroxytamoxifen has a low potency *in vivo* (but not *in vitro*) due to its catechol structure, which can be readily oxidized and conjugated thus allowing for rapid clearance. It has antiestrogenic activity (82). Metabolite E has substantial estrogenic activity and thereby potentially acts as a tamoxifen antagonist, although it has not been identified in any significant quantity in humans (85). Some metabolites have a decreased affinity for the ER, such as N–desmethyltamoxifen, N–didesmethyltamoxifen, and Metabolite Y. All of the dimethylamino side chains of these metabolites have been modified. N–desmethyltamoxifen and Metabolite Y also have lower antagonistic activity than tamoxifen (86,87).

Tamoxifen undergoes metabolic activation by the cytochrome P-450 (CYP) enzymes. These enzymes consist of several families of isozymes (88). Within hepatocytes, the enzymatic action of CYP 3A4 and CYP 2D6 convert tamoxifen to N–desmethyltamoxifen (89,90) and 4–hydroxytamoxifen (91,92), respectively. Other isozymes are believed to play a minor role, but these are the major isozymes. The formation of tamoxifen–N–oxide is catalyzed from tamoxifen in the liver primarily by microsomal flavin-containing monooxygenase (93).

1.2.3.1.3 Pharmacokinetics of Tamoxifen and Metabolites

Serum concentrations of tamoxifen have shown to be optimal just three hours after oral administration (94). Steady-state serum concentrations are achieved after four weeks of treatment. The half-life of tamoxifen in humans is around seven days (95). Steady-state concentrations for N–desmethyltamoxifen are achieved after eight weeks. The half-life for N–desmethyltamoxifen is thus assumed to be about 14 days (94). The long biological half-life of tamoxifen is attributed to a high level of plasma binding (probably to albumin) (96), and to enterohepatic recirculation (97). In serum, steady state concentrations of tamoxifen and N–desmethyltamoxifen have been shown to remain stable over many years of therapy (98).

In plasma, tamoxifen and N–desmethyltamoxifen have been found as the major metabolites, while 4–hydroxytamoxifen is found in low concentrations (99). N–Didesmethyltamoxifen has been found in serum (100). Some reports have found two minor metabolites in serum, Metabolites E and Y (100,101). Tamoxifen and its metabolites are excreted primarily by the biliary route. The highest proportions (25-50%) of tamoxifen and 4–hydroxytamoxifen are found in feces, with approximately 20% excreted into urine (102). Typically, the major metabolite found in urine is 4–hydroxytamoxifen (102).

1.2.4 Drug Excretion

Excretion occurs by a variety of pathways. The kidney, liver, and lungs are the primary organs. Drugs with molecular weights of approximately 300 amu are excreted largely into the urine, whereas drugs with higher molecular weights are excreted mainly into the bile first and then through the intestine (75). Once in the intestine, the drug can be excreted into the feces or it can be hydrolyzed back to the parent drug, absorb through the gut wall, and undergo enterohepatic circulation (103).

1.3 Analysis of Tamoxifen Metabolism

Gas chromatography with mass spectrometry (GC/MS) has traditionally been used to study drug metabolism, including tamoxifen metabolism (101,104). One challenge faced when using this technique is that sample derivatization is required for polar metabolites. High performance liquid chromatography (HPLC) with UV absorbance or fluorescence detection, utilized for drug metabolism studies, has routinely been used for the analysis of tamoxifen and its metabolites (105-112). High performance liquid chromatography with electrospray mass spectrometry (LC/ESI/MS) has been used for the analysis of tamoxifen and its metabolites (113,114). Recently, LC/ESI/MS has become the preferred method of analysis over GC/MS and conventional LC/MS for drug metabolism and pharmacokinetic research (103). Reasons for this include good sensitivity, reliability, specificity for a wide variety of compounds, and minimal sample handling requirements. Capillary electrophoresis (CE) with UV absorbance (115,116) or ESI/MS detection (117) has been used for the analysis of tamoxifen and its metabolites.

1.3.1 Capillary Electrophoresis

Capillary electrophoresis (CE) is an analytical technique in which ions separate within a capillary (typically 10-75 μm i.d.) due to a high electric field (typically 100–500 V/cm). Ions migrate through the capillary at different velocities based on their electrophoretic and electroosmotic mobilities (118). The velocity of an ion can be determined:

$$\text{velocity} = \frac{V(\mu_{eo} + \mu_{ep})}{L} \tag{1.1}$$

where

V is the applied voltage (V)

μ_{ep} is the electrophoretic mobility ($m^2V^{-1}s^{-1}$)

μ_{eo} is the electroosmotic mobility ($m^2V^{-1}s^{-1}$)

L is the capillary length (cm)

The electrophoretic mobility can be described in physical parameters:

$$\mu_{ep} = \frac{q}{6\pi\eta r} \tag{1.2}$$

where

μ_{ep} is the electrophoretic mobility ($m^2V^{-1}s^{-1}$)

q is the charge on the ion (C)

η is the viscosity of the solvent (cP)

r is the radius of the ion (m)

Electroosmotic mobility is described by the Helmholtz-Smoluchowski equation:

$$\mu_{eo} = \frac{\varepsilon\varepsilon_o\zeta}{\eta} \tag{1.3}$$

where

μ_{eo} is the electroosmotic mobility ($m^2V^{-1}s^{-1}$)

ε is the dielectric constant of the solvent (dimensionless)

ε_o is the permittivity of free space ($8.85 \times 10^{-12}\ C^2J^{-1}m^{-1}$)

ζ is the zeta potential (V)

η is the viscosity of the solvent (cP)

Note the following relationship:

$$\zeta \propto \frac{1}{\sqrt{I}} \tag{1.4}$$

where

ζ is the zeta potential (V)

I is the ionic strength (mol L^{-1})

Capillary electrophoresis is an efficient separation method because high voltages can be utilized. Efficiency is directly proportional to the running voltage:

$$N = \frac{(\mu_{ep} + \mu_{eo})\,V}{2D} \tag{1.5}$$

where

N is the number of theoretical plates (dimensionless); an expression of efficiency

μ_{ep} is the electrophoretic mobility ($m^2V^{-1}s^{-1}$)

μ_{eo} is the electroosmotic mobility ($m^2V^{-1}s^{-1}$)

V is the applied voltage (V)

D is the solute's diffusion coefficient (cm^2s^{-1})

There are however, practical limits to applying too high a voltage. A higher voltage produces more Joule heating, which leads to band broadening and a loss of resolution. It should be noted however, that there is less Joule heating in nonaqueous solutions compared to aqueous solutions, since the electric current is less in nonaqueous solutions.

1.3.1.1 CE Injection

An in-house constructed CE system, used in the following experiments, injects samples into capillaries electrokinetically, not hydrodynamically. Though not a major concern here, different ions usually load onto the capillary in varying amounts due to their different electrophoretic mobilities with electrokinetic injection. In electrokinetic injection, sample enters the capillary by electrostatic migration and the pumping action of electroosmotic flow. The quantity injected can be calculated:

$$Q = \frac{(\mu_{eo} + \mu_{ep})\, V\, \pi r^2\, C\, t}{L} \qquad (1.6)$$

where

Q is the quantity injected (g or moles)

μ_{ep} is the electrophoretic mobility ($m^2V^{-1}s^{-1}$)

μ_{eo} is the electroosmotic mobility ($m^2V^{-1}s^{-1}$)

V is the applied injection voltage (V)

r is the capillary radius (μm)

C is the analyte concentration (g mol $^{-1}$ or mol L^{-1})

t is the injection time (s)

L is the capillary length (cm)

The injection time and injection voltage are directly proportional to the amount of sample injected. The amount injected is important since too much sample in the capillary can cause sample overloading and thus decrease resolution. On the other hand, too small an amount injected causes sensitivity to decrease.

The quantity injected can also be estimated in terms of volume. An estimate to determine the volume injected is shown for a typical injection:

$$\begin{aligned} \mathrm{Vol_{inj}} &= \mathrm{Vol_{cap}}\left(\frac{t_{inj}}{t_m} \times \frac{V_{inj}}{V_m}\right) \\ &= \pi\,(50\ \mu\mathrm{m})^2\ (60\ \mathrm{cm})\left(\frac{5\ \mathrm{s}}{8\ \mathrm{min}} \times \frac{10\ \mathrm{kV}}{(20\mathrm{kV} - 4\ \mathrm{kV})}\right) \\ &= 30\ \mathrm{nL} \end{aligned} \tag{1.7}$$

where

$\mathrm{Vol_{inj}}$ is the volume injected

$\mathrm{Vol_{cap}}$ is the volume of the capillary

t_{inj} is the injection time

t_m is the migration time

V_{inj} is the injection voltage

V_m is the running voltage (applied voltage less ionspray voltage)

The reason this is only an estimate is because the injection voltage is not uniform. In the injections for these experiments, vials were filled with 20 µL for each run (20,000 nL), which is sufficient, even for multiple runs.

Nonaqueous solvents have fast rates of evaporation. Therefore, it is critical to know if enough solution would evaporate to significantly alter results, particularly with multiple injections from the same sample. Notwithstanding, vials were capped in these experiments immediately following each injection to prevent evaporation.

1.3.2 Electrospray Ionization

Electrospray is a soft ionization technique based on the application of a large electric field to nebulize solvent and allow ions, present in solution, to be transferred to the gas phase. The pseudomolecular ion $[M + H]^+$, is usually generated in electrospray (119). Adduct ions of NH_4^+, Na^+, or K^+ with the parent compound can also be formed. Ionspray is electrospray with nebulization assisted by pneumatics (120).

Four major processes take place in electrospray or ionspray: First, charges build up at the liquid surface causing a Taylor cone due to the electric field. As the cone becomes unstable, a liquid filament forms, and eventually separate charged droplets will form. Second, evaporation of the solvent causes the charged droplets to shrink in size, but not in charge. Third, when Coulombic forces within each droplet become sufficient to overcome surface tension, fission of the droplet occurs, and the offspring droplets repeat this disintegration process. Fourth, single or multiply charged gas phase ions are generated from extremely small droplets (121).

Electrospray is useful in drug metabolism because it permits the separation and ionization of polar nonvolatile or thermally labile compounds. Electrospray ionization was first demonstrated (122) on a quadrupole mass spectrometer, and has shown to be well suited to this type of mass spectrometry.

1.3.3 Mass Spectrometer

Mass spectrometers separate gaseous ions based on their mass-to-charge ratio and count these ions to provide mass spectra for the sample. The major components of a mass spectrometer include something to introduce the sample, an ion source, a mass analyzer, an ion detector, and a data system. In the experiments here, a PE/Sciex API 100 single quadrupole mass spectrometer was used to analyze samples. High performance liquid chromatography with a PE/Sciex API 3000 tandem mass spectrometer (MS/MS) was also used to analyze a few patients' samples. Sample was introduced into the mass spectrometer following CE separation by ionspray and ions were guided through the instrument with the use of ion optics. The ions come from the generation of gas–phase ions from the electrospray process. The mass analyzer is a quadrupole mass filter. The ion detector is a channel electron multiplier (CEM).

1.3.3.1 Ion Optics

Electrical fields established by multiple electrodes in the mass spectrometer move ions into the quadrupoles. Figure 1.2 shows the ion optics path of the API 100 MS and Table 1.4 gives standard voltage settings for these electrodes. Some of the main functions of these electrodes and other mass spectrometer elements follow. The curtain plate is maintained at a fixed voltage and separates sample flow from curtain gas flow. The curtain gas and the orifice are part of the atmospheric pressure region that transfers ions into the vacuum region. The curtain gas, high-purity nitrogen, prevents contamination of the ion optics by preventing sample and solvent from passing through the orifice, yet still permits the ions to enter the vacuum chamber. The curtain gas also provides a dry region for declustering solvent molecules from the ions. The orifice plate divides the atmospheric region from the differentially pumped interface region. This region is maintained at about 2 Torr. The orifice is the only access to the vacuum chamber through which all things, ions and a small volume of curtain gas, must pass. The skimmer provides a potential difference and with the orifice plate causes ion fragmentation. The focusing ring focuses ions through the skimmer into the first vacuum chamber (8×10^{-3} Torr). The ion path inside this chamber contains a RF-only quadrupole (Q0). QO transfers ions between the two vacuum chambers. The higher pressure region in Q0 allows ions to enter the mass filter region (Q1) with low axial and kinetic energies. The interquad lens (IQ1) helps transmission of ions into the second vacuum chamber (1.1×10^{-5} Torr), and into the Q1 mass filter quadrupole. By increasing the negative value of IQ1, a greater potential exists to draw ions into the Q1 region. The stubbies (ST),

Figure 1.2. Ion optics path of the API 100 MS.

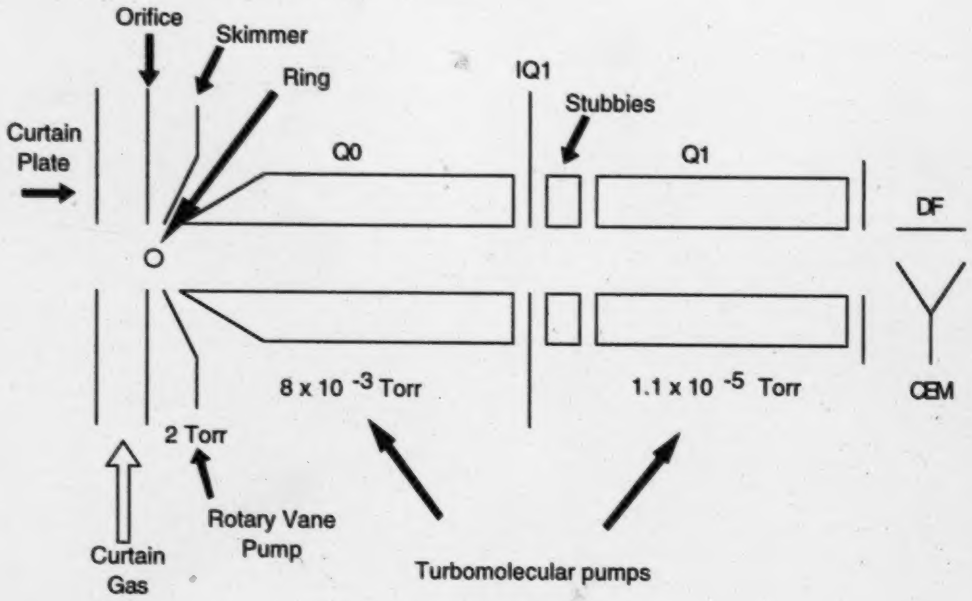

Orifice
Skimmer
Ring
IQ1
Stubbies
Curtain Plate
Q0
Q1
DF
8 x 10 -3 Torr
1.1 x 10 -5 Torr
CEM
2 Torr
Rotary Vane Pump
Curtain Gas
Turbomolecular pumps

Table 1.4. Standard voltage settings for ion optics of the API 100 MS.

Component	Identifier	Typical Voltage (V)
Curtain Plate	-	1000
Orifice Plate	OR	35
Focusing Ring	RNG	300
Quadrupole 0	Q0	-10
Interquad Lens 1	IQ1	-12
Stubbies	ST	-15
Deflector	DF	-200
Ion Detector	CEM	3000

transfer ions into Q1 by focusing the ions into the Q1 mass filter. Q1 is a mass analyzer filter that passes ions of the selected m/z range. DF is a fixed high voltage that deflects ions into the CEM. It is set according to the polarity of the ions to be analyzed. Lastly, the CEM counts ions. The CEM is described in detail in Section 1.3.3.3.

1.3.3.2 Quadrupole Mass Analyzer

Quadrupole mass analyzers use four cylindrical rods as electrodes to filter masses of ions based on their mass to charge ratio. Opposite rods are connected electrically, one pair having a positive variable DC source and the other having a negative variable DC source. Radio-frequency AC potentials are also applied to each pair of rods. Ions are accelerated by voltage potentials and injected into the space between the rods. For a particular field defined by the RF to DC ratio, ions within a m/z range will have stable paths through the rods, whereas all other ions will not be transmitted. These stable paths are defined by the Mathieu equations (123). Those ions that pass through the rods can then be detected.

The operator sets the mass range, and hence which ions will be detected. A single ion or a range of ions can be scanned. Other parameters that are set include the step size (in amu) of the increments in the mass range that are scanned, the dwell time, and the pause time. The dwell time is the time allotted for acquiring data at each step in the mass range. Pause time is a specified time for the computer to ensure all data have been collected before the next scan starts. Altering these parameters determines the scan speed. As the scan speed decreases, sensitivity can be increased.

In tandem mass spectrometry (MS/MS), there are three sets of quadrupoles. The first and third quadrupoles transmit only the selected ion and its selected fragment. The second set of rods acts as a collision cell for increased fragmentation of ions. A product ion scan is one where a single ion is transmitted through the first set of rods, and then in the third rod, a mass range is scanned. MS/MS is useful for determining structural information about the analyte due to characteristic fragmentation patterns and provides optimum sensitivity and selectivity.

1.3.3.3 Channel Electron Multiplier Detector

A channel electron multiplier is a small horn-shaped tube made of lead doped glass. Ions passing through the rods are drawn into the CEM by a fixed voltage on the DF plate. As ions collide with the CEM they produce a pulse of electrons, which cascades along the axis of the CEM due to another potential voltage. The electron pulse is collected and converted to a digital signal, which is counted to provide an ion count as a function of ion mass selected by the mass analyzer.

1.3.4 Interfacing CE to MS

The high efficiency of a CE separation can be coupled with the high sensitivity of mass spectrometry detection to give a powerful analysis (124,125). The interface between CE and MS should (125):

- maintain separation efficiency and resolution
- be sensitive, precise, and have a linear response
- maintain electrical continuity across the separation capillary
- cope with all eluents from CE separation
- provide efficient ionization from low flow rates for mass analysis

Electrospray Ionization is the preferred method for interfacing CE to MS, because more than any other method, it meets the above requirements.

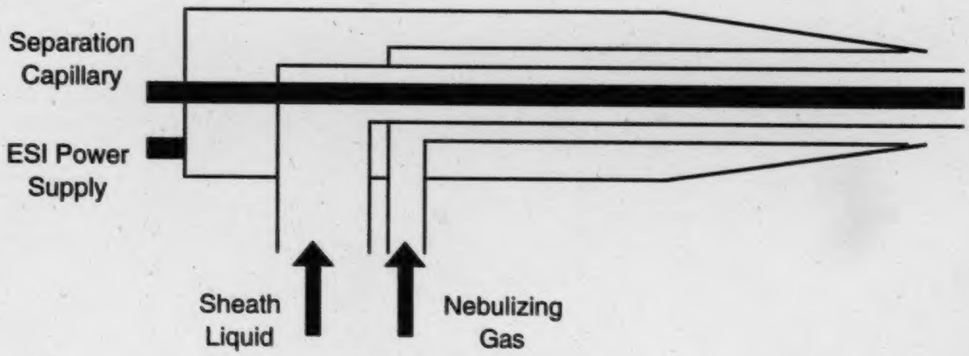
1.3.4.1 CE/ESI/MS

There are a number of electrospray interfaces used to combine CE to MS (126-129). The type of interface utilized in the following experiments (Figure 1.3) is known as the coaxial sheath-flow interface (126). Sheath liquid, with electrolytic content, is infused into the ESI source at a constant rate (1–4 μL/min). Sheath liquid surrounds the capillary and mixes with CE buffer at the end of the capillary, which is inserted 0.5–1.0 mm inside the tip of the ESI electrode, the electrode being positioned 0.5-1.0 mm outside the nebulizer tube. The mixing of sheath liquid and CE buffer provides the necessary electrical contact between the ESI needle and the CE buffer and also closes the CE circuit. CE/MS interfacing is complicated by the need to complete electrical paths for both the CE and the ESI systems. In the experiments here, typical applied voltages are 4 kV for ESI and 20 kV for the anodic capillary end. The actual voltage for CE separation would be 16 kV because the cathodic or terminal end of the capillary is not grounded, but instead is the same as the source for ESI voltage.

1.3.5 Nonaqueous CE/ESI/MS

Nonaqueous CE/ESI/MS has been proven to be exceedingly useful in pharmacological and clinical studies (130-137). In conventional CE, an aqueous electrolyte buffer is used to separate polar species. Nonpolar species can be separated with micellar electrokinetic chromatography (MEKC), which employs the use of surfactants (138). With mass spectrometry, the use of surfactants can foul the ion source and suppress analyte signals from ESI (137,139). Hydrophobic compounds, like tamoxifen, are not soluble in aqueous solutions, and thus cannot be analyzed with aqueous CE. Nonaqueous CE does not have these same constraints as conventional CE and still provides strong selectivity and separation potential. Nonaqueous CE consists of an electrolyte in an organic solvent or mixture of organic solvents.

Figure 1.3. Co-axial sheath-flow CE/MS interface to an ESI source.


Separation Capillary
ESI Power Supply
Sheath Liquid
Nebulizing Gas

A number of factors should be considered when nonaqueous CE is used (132). First, are the electrolyte and analyte soluble in the nonaqueous solution? Second, should one organic solvent be used or a mixture? If a mixture is used, what is the composition of that mixture? Third, what is the volatility of the nonaqueous solution? Nonaqueous solutions evaporate easily and experimental set-up should be designed to avoid evaporation. Fourth, are the solvents pure? Are they toxic? Considering such issues is necessary for a nonaqueous CE method to be validated and optimized.

Nonaqueous solvents can effect analyte migration times by altering physical properties of the solution (dielectric constants, viscosity, zeta potential, etc.) or altering chemical equilibrium properties that involve analyte and buffer electrolytes (acid-base reactions, etc.). Table 1.5 compares some parameters of nonaqueous CE to aqueous CE.

In addition to the parameters in Table 1.5, when using CE/ESI/MS, the lower currents in nonaqueous CE are more comparable than those of aqueous CE to currents generated in the ESI interface and therefore the sensitivity and stability of the system is higher with nonaqueous CE. These lower current values also give an increased efficiency in separations due to less Joule heating. However, some have argued though that the smaller heat capacity and lower thermal conductivity of non-aqueous solutions may counter the reduction in heat from less Joule heating (135). Also, nonaqueous solvents evaporate easier than aqueous solvents and thus ionization in the electrospray process increases.

Nonaqueous CE/ESI/MS has previously been used for the analysis of tamoxifen and metabolites (117). In this case however, surfactants were used. Also, human samples of tamoxifen and its metabolites were not analyzed, but rather products formed after incubation of tamoxifen with mouse hepatocytes.

Table 1.5. Comparison of parameters in nonaqueous CE and aqueous CE (132).

Parameter	Comparison
Selectivity	Various organic solvents and combinations of different compositions in mixtures can be used instead of water to alter relative migration times of analytes due to changes in: •electrophoretic mobility (see Equation 1.2) •dissociation constants of acids/bases ('levelling effect' of water is absent or at least weaker in organic solvents
Efficiency	•Currents in nonaqueous buffer are lower, allowing use of higher electrolyte concentrations, resulting in greater differences in ionic strengths between samples and buffers, and thus higher efficiency in nonaqueous CE •Better peak shapes with hydrophobic compounds due to decreased interactions with capillary wall are seen in nonaqueous CE
Electroosmotic Flow	•Less in nonaqueous CE (except acetonitrile) solutions (see Equation 1.3)
Analysis Time	•Shorter in nonaqueous due to increased separation voltages; voltages can be increased due to lower current in nonaqueous CE •Longer in nonaqueous CE from lower electroosmotic flows if separation voltages are unchanged from aqueous analysis

1.4 Statistical tests

A number of statistical tests were used to determine relationships between patient clinical characteristics and tamoxifen metabolic profiles. The statistical package used was SAS 6.12 by SAS Institute Inc. (Cary, NC, U.S.A). The tests and some of the terms used with those tests are briefly described below (140).

Probability (p) provides a quantitative description of the likely occurrence of a particular event. Probability is conventionally expressed on a scale from 0 to 1. A significant event has a probability close to 0; an insignificant event has a probability close to 1. Significant probabilities in these experiments were determined from p values of less than 0.05.

Correlation is a measure of the relation between two or more variables. Correlation coefficients (R) can range from -1 to 1. The value of -1 represents a perfect negative correlation while a value of 1 represents a perfect positive correlation. A value of 0 represents a lack of correlation. The most widely used type of correlation coefficient is Pearson, also called linear correlation. The correlation coefficient determines the extent to which values of two variables are proportional to each other. The correlation coefficient does not depend on the specific measurement units used. Proportional relationships are linearly related; that is, the correlation is high if it can be approximated by a straight line (sloped upward or downward). This line is called the regression line or least squares line, because it is determined such that the sum of the squared distances of all the data points from the line is the lowest possible. Pearson correlation assumes that the two variables are measured on at least interval scales.

The Spearman rank correlation coefficient can be thought of as the regular Pearson correlation coefficient, except that the Spearman R is computed from ranks. Spearman R assumes that the variables under consideration were measured on at least an ordinal (rank order) scale. That is, the individual observations (cases) can be ranked into two ordered series.

The Chi-square test allows one to decide whether observed frequencies are essentially equal to or significantly different from frequencies predicted by a theoretical model. The outcome decides whether or not two variables are independent.

The t-test is the most commonly used method to evaluate the differences in means between two groups. The groups can be independent or dependent. Theoretically, the t-test can be used even if the sample sizes are small (n=10) as long as the variables are approximately normally distributed.

Multivariate analysis means using many variables to forecast, predict, or understand a situation. For instance, to predict the likelihood of just one particular patient characteristic correlating to the concentration of a tamoxifen metabolite, one should consider not just that particular characteristic, but others that may also affect metabolite concentration. To get a more accurate picture, a wide range of factors must be considered. The computational problem that needs to be solved in multivariate regression analysis is to fit a straight line to a number of points.

Cox regression analysis (141) is used for survival time analysis of patients. Given patient survival times, final status (alive or dead), and one or more variables, the analysis produces a baseline survival curve, variable coefficient estimates with their standard errors, risk ratios, 95% confidence intervals, and significance levels. The analysis here takes the survival times of a group of advanced stage breast cancer patients and generates a survival curve, which shows how many of the patients remain alive over time. The survival time is defined as the length of the interval between diagnosis and death. The major mathematical complication with this analysis is that the data is analyzed while the patients are still alive (i.e. the fate of all patients is unknown).

1.5 Summary of Chapters

Chapter 1 was an introduction to the principles that will be discussed in the forthcoming chapters. In Chapter 2, a method for the analysis of tamoxifen and its metabolites using nonaqueous CE/ESI/MS is discussed. The method is validated with respect to within-day precision and to between-day precision. Calibration curves are constructed from the analysis of standards and absolute concentrations are determined in a few patients. LC/ESI/MS is also used for analysis. The stability of samples within one day and over a larger period and relative comparisons between patients is also considered. In Chapters 3 and 4, the analysis of tamoxifen and its metabolites is performed in urine and serum samples respectively. In these two chapters an investigation as to how tamoxifen is metabolized in humans is conducted and the hypothesis of differential metabolism in individual patients is confirmed. Relationships between patient samples and tamoxifen metabolic profiles and clinical characteristics are explored. In Chapter 5, two in vitro tamoxifen experiments are performed. In the first, tamoxifen and its acid hydrolysis products are separated and tentatively identified by nonaqueous CE with thermo–optical absorbance CE/ESI/MS. In the second experiment, the transformation of tamoxifen at 37 °C in synthetic gastric fluid is analyzed by high performance liquid chromatography with triple quadrupole mass spectrometry. Chapter 6 gives a summary of the thesis and discusses future work.

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Chapter 2

Development of a Nonaqueous CE/ESI/MS Method for the Analysis of Tamoxifen and Its Metabolites in Patient Samples

2.1 Introduction

Several analytical techniques including high performance liquid chromatography (HPLC) with UV absorbance or fluorescence detection are used for routine analysis of tamoxifen and its metabolites (1-8). Gas chromatography with mass spectrometry detection (GC/MS) has also been used (9,10), although this method has the disadvantage of requiring sample derivatization of polar metabolites. Recently, high performance liquid chromatography with electrospray mass spectrometry (LC/ESI/MS) has become the preferred method of analysis over GC/MS and conventional LC/MS for drug metabolism and pharmacokinetic research (11). Reasons for this include good sensitivity, reliability, specificity for a wide variety of compounds, and minimal sample handling requirements. LC/ESI/MS has been used for the analysis of tamoxifen and its metabolites (12,13). Capillary electrophoresis (CE) has been used for the analysis of tamoxifen and its metabolites (14-16). Nonaqueous CE with ESI/MS has already been used for the analysis of tamoxifen and metabolites (16), however surfactants were used. Surfactants can cause fouling of the ion source and analyte signals can be suppressed (17). Further, these authors analyzed tamoxifen and its metabolites formed after incubation of tamoxifen with mouse hepatocytes.

In this chapter, a nonaqueous CE/ESI/MS method was developed for the analysis of tamoxifen and its metabolites in human urine and serum samples, without the use of surfactants. The analytical method was optimized with respect to CE separation and mass spectrometer detection. The method was validated with respect to within-day and between-day precision.

2.2 Experimental

2.2.1 Reagents

Tamoxifen was obtained from Sigma (St. Louis, MO, U.S.A.) and 4–hydroxytamoxifen (mixture of E and Z isomers and Z isomer only) from Research Biochemical International (Natik, MA, USA). N–desmethyltamoxifen was obtained from Sciex (Concord, ON), and 4–hydroxy–N–desmethyltamoxifen was obtained from Mr. Ken Roberts at Iowa State University. Tamoxifen–N–oxide (18), Metabolite E (19-22), and Metabolite Y (23,24) were synthesized by following procedures found in the literature. The general workup procedure involved quenching of the reaction mixture with water and repeated extractions of the aqueous layer with ether. The organic layer was pre-dried by washing with brine, and was allowed to stand over sodium sulfate or magnesium sulfate. The hydrated salt was removed by gravity filtration, and the organic solvent was removed using a rotary evaporator under reduced pressure. The crude product was purified by flash chromatography. Purity was checked with TLC, and the product was then recrystallized. Intermediate products were identified by ^{1}H NMR (300 MHz, Bruker, see Figure 2.1 spectra) and IR ($CDCl_3$ cast), and the metabolites were identified additionally using ^{13}C NMR (300 MHz, Bruker) and electron impact mass spectrometry.

Because standards for 3,4–dihydroxytamoxifen and N–didesmethyltamoxifen were not obtained, in-source collision induced dissociated (CID) was utilized on patient samples to obtain higher fragmentation spectra to tentatively identify these metabolites. Orifice voltages were increased from typical usages of about 40 V in regular analyses to 125 V for in-source CID analyses.

HPLC–grade methanol and acetonitrile were purchased from Caledon (Georgetown, ON) and EM Science (Gibbstown, NJ, U.S.A.), respectively. Ammonium acetate was purchased from ACP Chemicals (Montreal, PQ). Tamoxifen and metabolite stock solutions were prepared by dissolving directly into methanol. Standard solutions (0.001-1 μg/mL) were then prepared by serial dilution with methanol.

2.2.2 Instrumentation

2.2.2.1 Capillary Electrophoresis

An inhouse constructed CE system was used for separations. Briefly, a spring-loaded plexiglass carousel (10 cm diameter) with 10 wells, which hold 600 μL vials, was used. A platinum wire electrode (approximately 2.5 cm long) connected to a plexiglass platform supplied the high voltage (Spellman high voltage DC supply, model #RHR30N10CR/RVC(10), Bronx, NY). The capillary was placed through a hole in the platform into the buffer vial, about 1 mm from the platinum wire electrode, prior to sample injection. For injections, a plunger was pushed down to manually move the carousel from the buffer vial to the sample vial and then back to the buffer vial. An interlock safety device, located on the opposite side of the carousel from the platinum wire, would slide into troughs, which were located between the wells, allowing the high voltage connection to occur only when the carousel was in position. All runs were done at ambient temperature. Untreated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, U.S.A.) with 60 cm x 50 μm i.d. x 150 μm o.d. dimensions were used for separations. Fresh running buffers were used to flush the capillary before and between runs. Flushing was performed manually with a 1.0 mL plastic syringe fused at the tip with a small, narrow glass piece in the shape of a cone, which was prepared from the end of a Pasteur pipet.

2.2.2.2 Electrospray Ionization Mass Spectrometry

A PE/Sciex API 100 LC single quadrupole mass spectrometer equipped with an ion spray source was used to analyze eluents. The CE column was inserted 0.5-1.0 mm inside the tip of the ESI electrode, and the electrode was 0.5-1.0 mm outside the nebulizer tube.

A Harvard Apparatus (South Natick, MA, U.S.A.) low-pressure syringe infusion pump was used to pump methanol, the coaxial sheath liquid. The flow rate was 2.0 μL/min through a capillary of 60 cm x 50 μm i.d. x 150 μm o.d. dimensions. Positive ion mode was used for all measurements. Ion spray voltages ranged from 3500 to 4600 V, orifice voltages from 10 to 25 V, ring voltages from 150 to 260 V, nebulizer gas flow rates from 0.03 L/min (NEB 0) to 0.67 L/min (NEB 1), curtain gas flow from 0.95 L/min (CUR 8) to 1.08 L/min (CUR 9), and CEM voltages from 2600 to 3000 V. A working pressure of 1.1 x 10^{-5} Torr was maintained in the analyzer chamber. During sample injections, the ionspray voltage was set to 0 and the nebulizer gas flow rate was set to 0.03 L/min (NEB 0). For data acquisition the mass to charge range was 50-800 amu, step size was 1.0 amu, scan speed was 1.13 s, dwell time was 1.5 ms, and pause time was 3.0 ms. Electropherograms were acquired by a LC Tune software (PE Sciex, Concord, ON), and then processed by Multiview 1.2 software from Sciex.

2.2.3 Experimental Procedure

2.2.3.1 Separation Conditions

Standards of the E and Z isomers of 4–hydroxytamoxifen were separated by using different concentrations of ammonium acetate in different mixtures of methanol and acetonitrile to determine optimal separation conditions. Optimal injection time, injection voltage, and running voltage were determined by varying these parameters while separating the isomers of 4–hydroxytamoxifen in the optimal solvent mixture and electrolyte concentration.

2.2.3.2 Method Validation

To validate these nonaqueous CE/ESI/MS conditions with human samples, the following were considered: First, metabolite migration times in 47 urine samples were compared. Second, one particular patient's urine sample was analyzed seven different times intermittently over 13 hours to determine the CE/ESI/MS repeatability. Third, absolute concentrations of tamoxifen metabolites analyzed on two different days were determined in six patients. Calibration curves were constructed using N–desmethyltamoxifen, 4–hydroxytamoxifen, and tamoxifen standards. R^2 values were calculated. Fourth, another analytical technique, LC/ESI/MS, was used to analyze and identify tamoxifen metabolite peaks.

2.2.3.3 Other Experimental Considerations

In addition to this, other factors were considered: First, the stability of samples was investigated. The effects of light and temperature on patient samples over a one-day period were determined. Three different samples were analyzed three times throughout the day. One sample was kept at room temperature and exposed to room light. Another was kept in ice and exposed to room light. The last was kept in the refrigerator at 4°C, unexposed to light. Also, to determine whether samples were stable and the anaylsis was reproducible over a longer period of time, a number of the same samples were analyzed weeks apart. Second, to ensure accurate comparisons between patients, corrections were made to the measured signal for each metabolite. The peak height for each metabolite was divided by the peak height of the internal standard. For urine an additional correction was made by dividing by creatinine concentration (this is further explained in section 3.2.6).

2.3 Results and Discussion

2.3.1 Metabolite Identification

2.3.1.1 Identification of Metabolites by using Standards Obtained

Tamoxifen, N–desmethyltamoxifen, 4–hydroxytamoxifen, and N–desmethyl–4–hydroxytamoxifen were all identified in patient samples by matching migration times of a standard peak to a peak in patient samples. Spiking patient samples with standards also verified metabolite identification. Tamoxifen, N–desmethyltamoxifen, and 4–hydroxytamoxifen were found in urine and serum samples. N–desmethyl–4–hydroxytamoxifen was found in serum, but not urine samples.

2.3.1.2 Identification of Metabolites using Synthesized Standards

Tamoxifen–N–oxide, Metabolite E, and Metabolite Y were synthesized by Grace Chan. Each of these compounds was analyzed by ^{1}H NMR, IR, ^{13}C NMR, and electron impact mass spectrometry for identification purposes. An example of the ^{1}H NMR spectra for tamoxifen–N–oxide is shown in Figure 2.1. Table 2.1 gives the interpretation for these spectra (see Table 1.3 for structure of tamoxifen–N–oxide). The integration for the multiplet at 7.25 ppm is higher than expected due to the presence of residual chloroform in the deuterated solvent. The chloroform peak appears at 7.26 ppm.

Figure 2.1. ^{1}H NMR spectra for synthetically prepared tamoxifen–N–oxide.

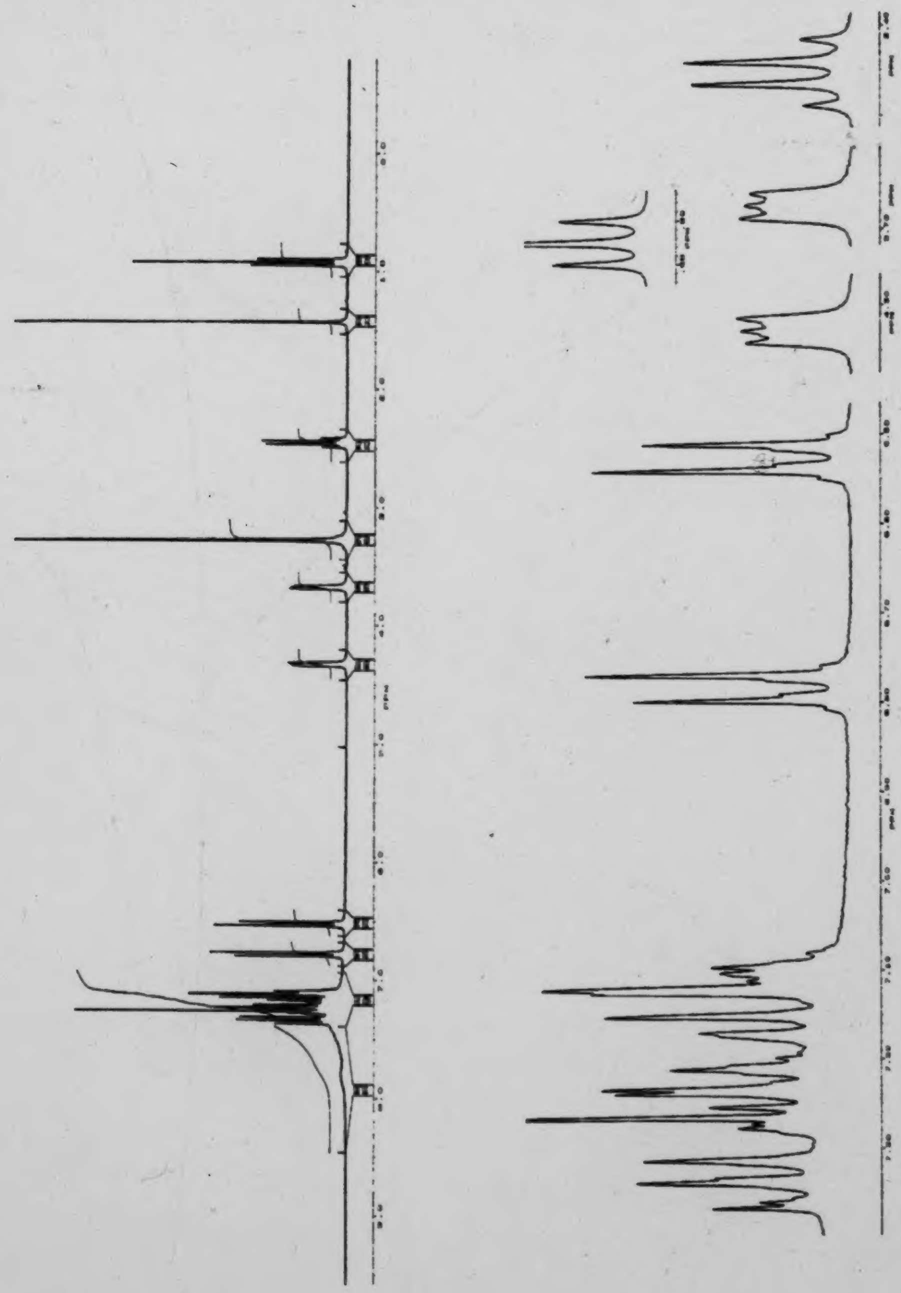


Table 2.1. ^{1}H NMR spectra interpretation for synthetically prepared tamoxifen–N–oxide.

Chemical Shift (ppm)	Multiplicity	Number of Neighbouring Hydrogen(s)	Theoretical Assignment	Actual Integration	Assignment
0.95	triplet	2	3	3.0	$-CH_2CH_3$
1.45	singlet	0	-	-	residual H_20
2.45	quartet	3	2	1.9	$-CH_2CH_3$
3.30	singlet	0	6	6.0	$-N(CH_3)_2$
3.65	triplet	2	2	1.9	$-NCH_2$
4.35	triplet	2	2	1.9	$-OCH_2$
6.55	doublet	1	2	2.1	ArH
6.80	doublet	1	2	2.3	ArH
7.25	multiplet	-	10	14.8	ArH

The peak in the extracted ion electropherogram (m/z 388) for the synthetically prepared standard of tamoxifen–N–oxide migrated at the same time as a peak in the extracted ion electropherograms (m/z 388) for urine sample extracts. Spiking experiments further confirmed that this peak in urine samples was tamoxifen–N–oxide. No tamoxifen–N–oxide was found in serum samples.

Metabolite Y was identified using the same analytical methods as tamoxifen–N–oxide. The synthesis product was a mixture of isomers. The peak for this standard in the CE/ESI/MS extracted ion electropherogram (m/z 345) did not match the migration time of other m/z 345 peaks in patient samples. Therefore, the m/z 345 peaks in patient samples remain unidentified.

Metabolite E was identified using the techniques listed and product was also a mixture of isomers. This standard showed no peaks from the extracted ion electropherogram (m/z 301) analyzed by CE/ESI/MS. Therefore, the m/z 301 peaks in patient samples remain unidentified.

2.3.1.3 Identification of Metabolites by using In-source Collision–Induced Dissociation

Electrospray ionization is a soft ionization technique meaning little fragmentation occurs. One advantage of electrospray is being able to easily identify compounds due to a single peak from the parent ion. This can be a disadvantage though when ion fragmentation is desirable. Ion fragmentation is useful for helping determine structural information. Although electrospray mass spectra usually do not contain ion fragments, it is possible to obtain some fragmentation simply by changing the orifice voltage so that the potential difference between the orifice and the skimmer increases. This has often been called in-source collision–induced dissociation (in-source CID). In-source CID increases the energy given to the ion during multiple collisions with neutral, residual gas molecules. An increase in energy results in an increase in fragmentation. Because standards for

3,4–dihydroxytamoxifen and N–didesmethyltamoxifen were not obtained, in-source collision induce dissociated (CID) was utilized on patient samples to obtain higher fragmentation spectra to tentatively identify these metabolites.

A number of experiments were performed to determine the optimal orifice voltage for in-source CID. At higher orifice voltages (> 150 V), there were many fragments peaks in the spectrum. The parent ion peak usually could not be seen. At lower orifice voltages (< 75 V), there were few peaks in the spectrum other than the parent ion; fragmentation was weak, resulting in a lack of structural information. It was determined that an optimal orifice voltage of 125 V in most cases (90 V was used for tentative identification of 3,4–dihydroxytamoxifen in Figure 2.4), gave sufficient structural information. With tamoxifen metabolites at this voltage, there were usually two major peaks and a few other minor peaks in spectra. This information was sufficient for tentative structural identification.

Figure 2.2 shows extracted ion electropherograms and the in–source CID spectra of three standards, N–desmethyltamoxifen, tamoxifen, and 4–hydroxytamoxifen. N–desmethyltamoxifen, tamoxifen, and 4–hydroxytamoxifen are well separated. Note that the spectra are similar for these three standards. The major peaks are the parent ion (m/z 358, 372, and 388 respectively) and the amino side chain without oxygen (m/z 58, 72, and 72 respectively).

Figure 2.3 shows the extracted ion electropherogram (m/z 344) of N–didesmethyltamoxifen in a patient's serum sample and the in-source CID spectrum of its peak. N–didesmethyltamoxifen, found in serum, but not urine, is tentatively identified using in-source CID. Included in this figure is the extracted ion electropherogram (m/z 358) of N–desmethyltamoxifen from the same sample and the in-source CID spectra of its peak. N–desmethyltamoxifen migrates close to N–didesmethyltamoxifen but does not overlap. This spectrum is similar to the standard N–desmethyltamoxifen spectrum in Figure 2.2.

Figure 2.2. Extracted ion electropherograms of N–desmethyltamoxifen, tamoxifen, and 4–hydroxytamoxifen with in-source CID spectra of peaks from standard samples.

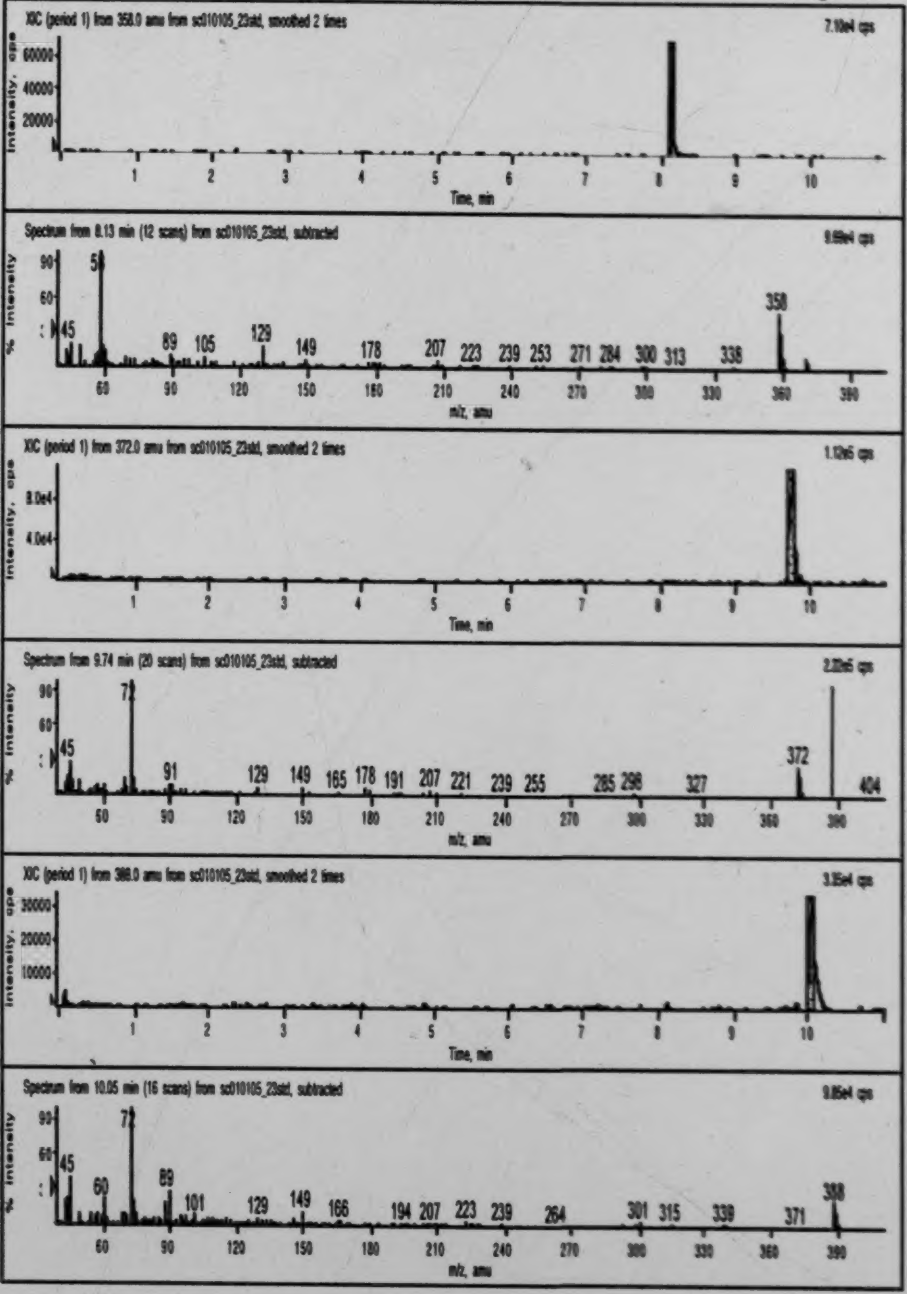


Figure 2.3. Extracted ion electropherograms of N–didesmethyltamoxifen and N–desmethyltamoxifen with in-source CID spectra of peaks from a patient's serum sample.

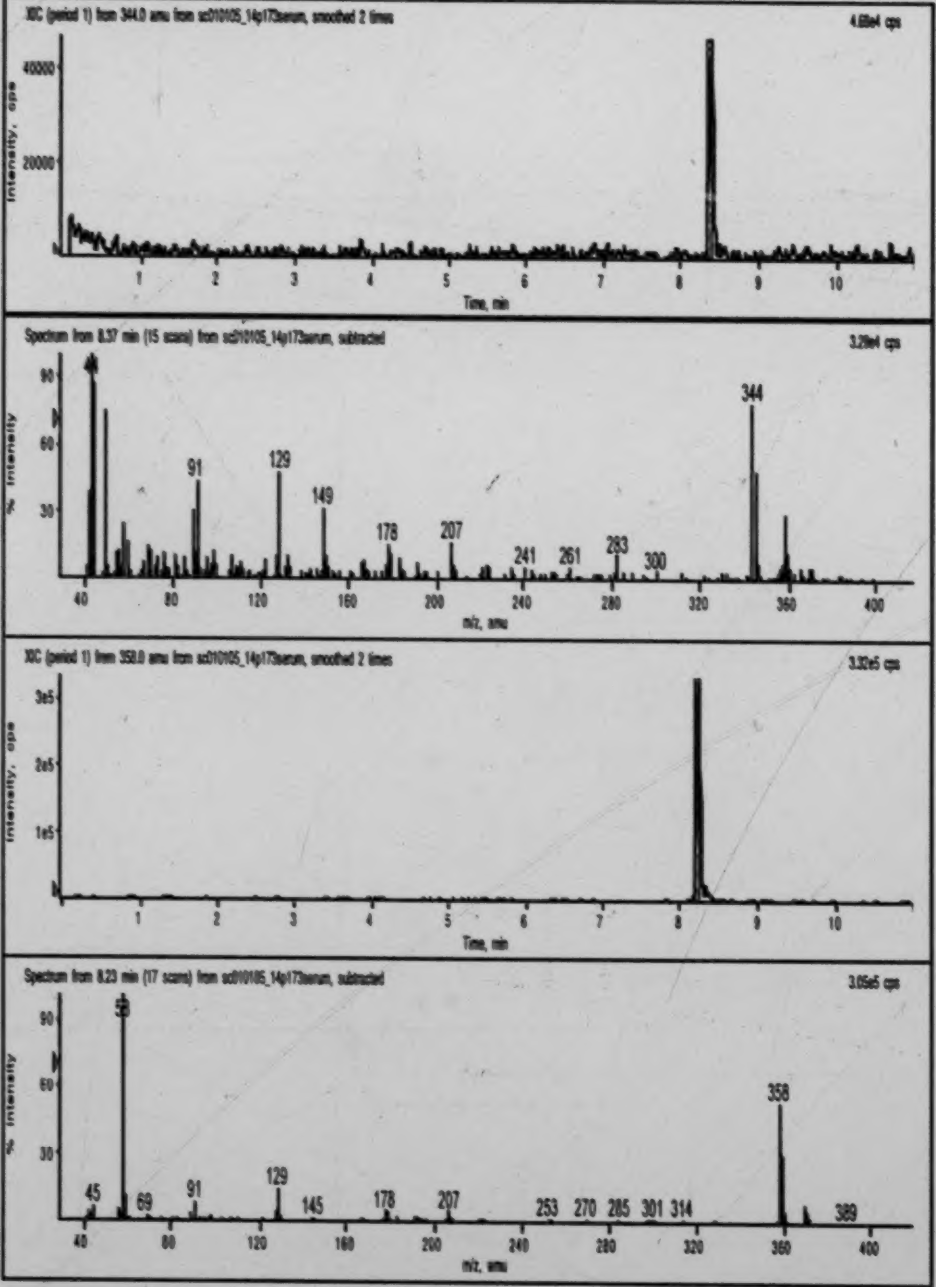


Figure 2.4. Extracted ion electropherogram of 3,4–dihydroxytamoxifen with in–source CID spectrum of peak from a patient's urine sample. 3,4–Dihydroxytamoxifen, found in urine is tentatively identified using in-source CID. In this particular sample, the m/z 404 peak had low intensity. Orifice voltage was 90 V.

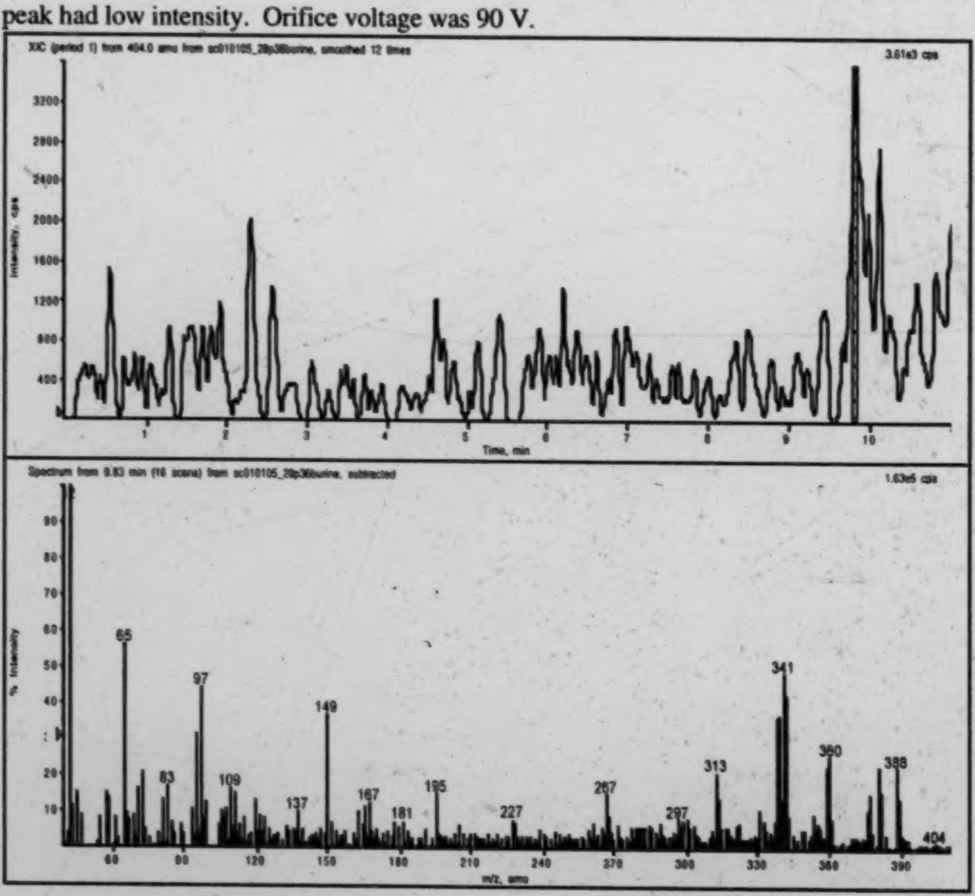
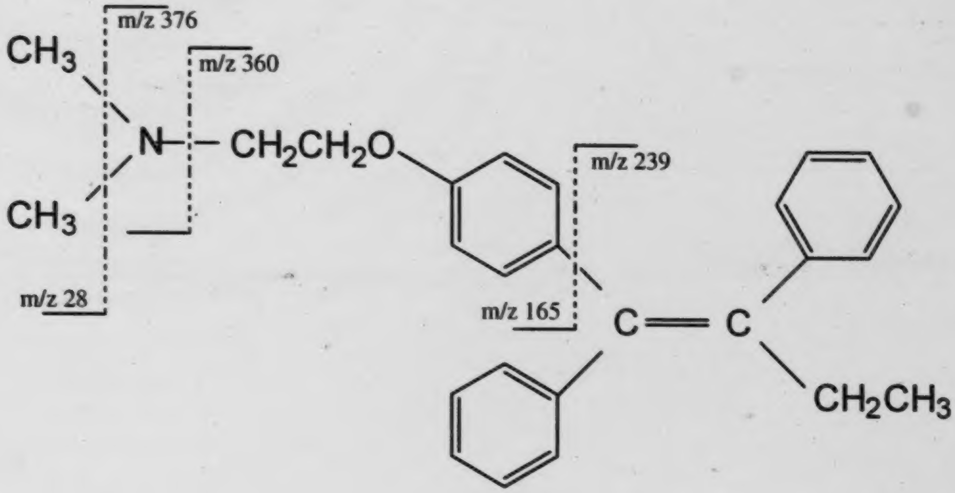


Figure 2.4 shows the extracted ion electropherogram of 3,4–dihydroxytamoxifen (m/z 404) from a patient's urine sample and the in–source CID spectrum of its peak. 3,4–Dihydroxytamoxifen, found in urine, but not serum, is tentatively identified using in-source CID. In this particular sample, the m/z 404 peak had low intensity. Therefore the orifice voltage was decreased to 90 V instead of 125 V. Higher orifice voltages increase fragmentation, but the total ion signal and thus the extracted ion signal decrease. One problem for this sample, with this method of identification, is that tamoxifen–N–oxide co–elutes with 3,4–dihydroxytamoxifen and therefore some spectrum peaks can be attributed to tamoxifen–N–oxide. Notwithstanding, some of the possibilities for these peaks are found in Figure 2.5. Tamoxifen and 4–hydroxytamoxifen have the same amino side chain as 3,4–dihydroxytamoxifen. Yet note that the m/z 72 peak in 3,4–dihydroxytamoxifen is not strong compared to the same peak in the in-source CID spectra of standard (Figure 2.2). One reason for the weaker peak signal at m/z 72 in 3,4–dihydroxytamoxifen may be due to the lower orifice voltage used.

2.3.2 Optimization of Separation Conditions

Being able to extract a single-ion signal from the total ion current may cause one to wonder why a separation is necessary when using mass spectrometry. There are several reasons why CE, as a separation technique, opposed to direct infusion of samples into the mass spectrometer was used here. First, some tamoxifen metabolites have the same molecular weight (such as tamoxifen–N–oxide and 4–hydroxytamoxifen) and without separation between such metabolites, identification would be impossible. Second, mixture components show a more uniform response after CE separation (25). Third, fragmented metabolites from the electrospray process, although rare, may have the same molecular weight as a different metabolite, in which case separation would be needed for identification. An example of this is N–didesmethyltamoxifen ($M + H^+ = 344$) and N–desmethyltamoxifen ($M + H^+ = 358$). The extracted ion electropherograms of m/z

Figure 2.5. Possible ion fragmentation assignments for 3,4–dihydroxytamoxifen found in patient's urine sample.


m/z 376
m/z 360
m/z 239
m/z 28
m/z 165
CH3
CH3
N
CH2CH2O
C
C
CH2CH3

358 and m/z 344 each give a single peak at approximately the same migration time. One may erroneously conclude that because of the similar migration times, the peak at m/z 344 is an N-desmethyltamoxifen fragment, having lost a methyl group from the parent ion. These peaks (m/z 358 and m/z 344) consistently give different migration times, albeit only slightly. In addition, the N–desmethyltamoxifen standard, at low orifice voltage conditions, had no fragments at m/z 344. In this case, the separation of N–desmethyltamoxifen and N–didesmethyltamoxifen (the metabolite at m/z 344) as well as the standard helped to identify N–didesmethyltamoxifen.

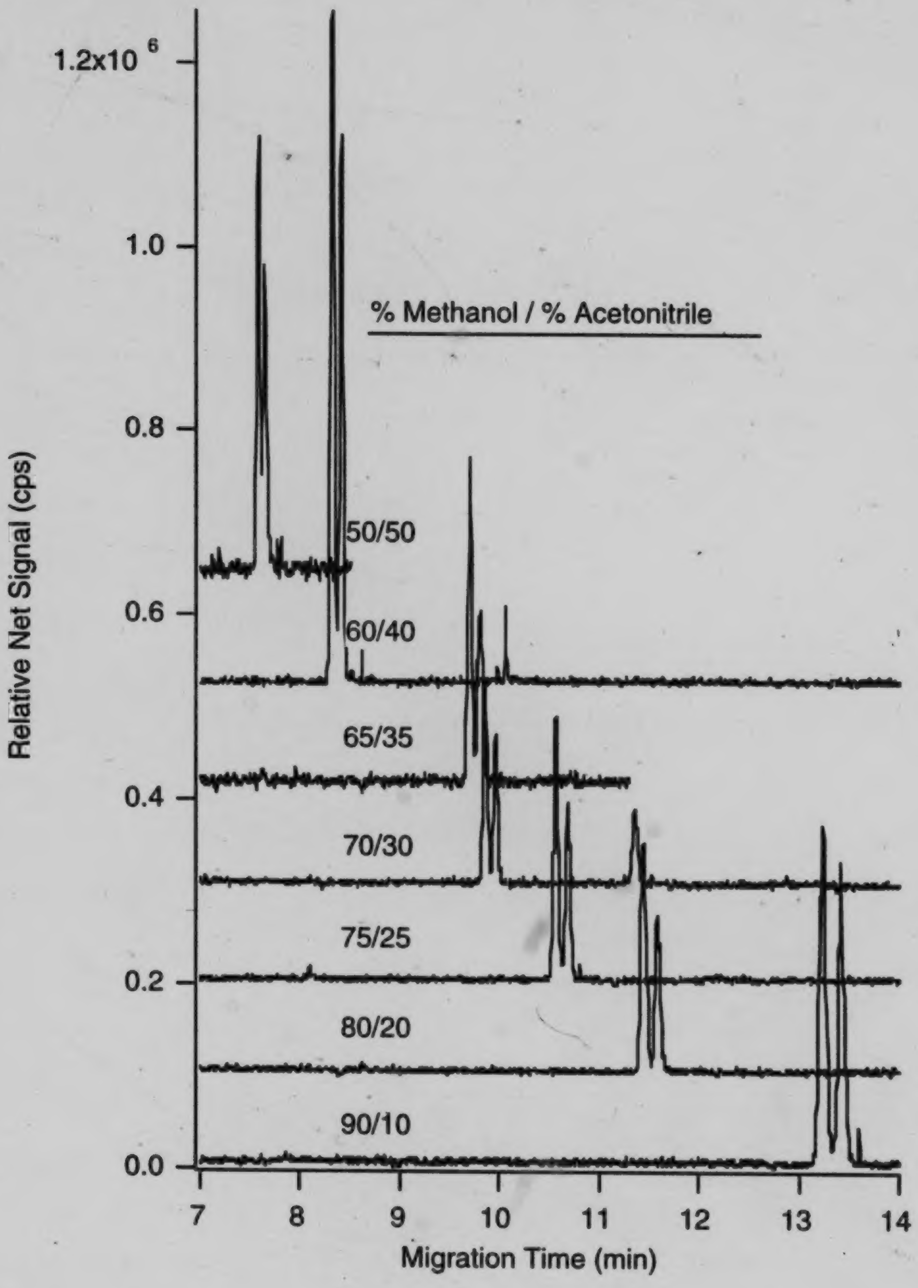
By using CE separation, selectivity can be altered. To increase selectivity in conventional capillary electrophoresis, many parameters can be varied. For instance, changing the pH or adding surfactants or other selectors such as cyclodextrins can increase selectivity. In nonaqueous media however, simply changing the organic solvent or varying the proportion of two solvents can optimize selectivity. In addition, the electrolyte and its concentration in solvent can effect selectivity.

2.3.2.1 Solvent

Different solvent systems have been used to separate tamoxifen metabolites including the use of organic modifiers and various electrolytes (14,16). One of these systems employed a methanol/acetonitrile mixture as the solvent with ammonium acetate as the electrolyte (15). Figure 2.6 shows electropherograms where the 4–hydroxytamoxifen isomers are separated using various mixtures of methanol and acetonitrile.

Migration time increased with increasing amounts of methanol. When 50% methanol / 50% acetonitrile was used, the migration time for the isomers was about 7.5 minutes. In CE, the Z isomer of 4–hydroxytamoxifen elutes before the E isomer. Migration time increases proportionally to the increased percentage of methanol used in the buffer. With a 90% methanol / 10% acetonitrile mixture, migration time was over 13 minutes. Although migration time was faster when less methanol was used, resolution

Figure 2.6. Extracted ion electropherograms (m/z 388) of the E and Z isomers of 4–hydroxytamoxifen in various mixtures of methanol/acetonitrile with 20 mM ammonium acetate as electrolyte.



between the isomers decreased. For the 50% methanol / 50% acetonitrile mixture, the separation resolution is calculated:

$$R = \frac{t_{r1} - t_{r2}}{\frac{1}{2}(w_2 + w_1)} \tag{2.1}$$

$$R = \frac{7.64 \text{ min} - 7.57 \text{ min}}{\frac{1}{2}(0.072 \text{ min} + 0.072 \text{ min})}$$

$$R = 0.87$$

where

R is the resolution (dimensionless)
t_{r2} is the migration time of (E)–hydroxytamoxifen
t_{r1} is the migration time of (Z)–hydroxytamoxifen
w_2 is the full width of the (E)–hydroxytamoxifen peak
w_1 is the full width of the (Z)–hydroxytamoxifen peak

For the 90% methanol / 10% acetonitrile mixture, resolution is also calculated:

$$R = \frac{t_{r1} - t_{r2}}{\frac{1}{2}(w_2 + w_1)}$$

$$R = \frac{13.43 \text{ min} - 13.24 \text{ min}}{\frac{1}{2}(0.136 \text{ min} + 0.136 \text{ min})}$$

$$R = 1.44$$

The 70% methanol / 30% acetonitrile mixture was decided as optimal because separation was fast (about 10 minutes) and resolution was sufficient (R=1.29) for the 4–hydroxytamoxifen isomers.

When comparing the migration times of the 4–hydroxytamoxifen isomers between electropherograms in Figure 2.6, those with a higher methanol content in the buffer had a slower velocity. Velocity (see Equation 1.1) is dependant on the electrophoretic mobility and electroosmotic mobility, which in turn are affected by other variables (see Equations 1.2 and 1.3, respectively). Two of these variables are the viscosity and the dielectric constant. The viscosity and dielectric constant values for methanol and acetonitrile are shown in Table 2.2. In the 90% methanol / 10% acetonitrile mixture, the methanol content is higher than any of the other buffers used in Figure 2.6. Therefore, the overall ε of the mixture in this buffer is lower, and the overall η is higher than the other buffers used in Figure 2.6. The μ_{eo} of the 90% methanol / 10% acetonitrile mixture, in turn, is lower than any of the other buffer mixtures, and hence, the migration time of the 4–hydroxytamoxifen isomers in this buffer is longer than any others.

The zeta potential is often used to qualitatively describe electroosmotic mobility. However, it has been shown quantitatively that the zeta-potential in acetonitrile without ionic additives is higher than in methanol (26,27). Therefore, changes in the overall zeta potential as the composition of a methanol/acetonitrile mixture varies would effect μ_{eo} the same way that changes in the overall ε or η would.

Methanol is an excellent solvent to use in ESI/MS because it has a low surface tension and thus allows droplet breakup and evaporation, which is critical to the electrospray process. It has a relatively high dielectric constant and solvation occurs due to the formation of hydrogen bonds. Methanol is capable of autoprotolysis and will accept and donate protons. Intermolecular hydrogen bonding between methanol molecules decreases upon adding a second solvent, in this case acetonitrile. Acetonitrile is a good additive because it also has a relatively high dielectric constant, even slightly higher than that of methanol, and can accept protons. Unlike methanol, acetonitrile is usually classified as a non–hydrogen bond donor because it is aprotic, making it a less suitable solvent for

Table 2.2. Some physical properties of methanol and acetonitrile.

Solvent	ε	η (cP)
Methanol	32.7	0.55
Acetonitrile	37.5	0.38

electrolytes. With the presence of methanol in the mixture though, electrolyte solubility is not a concern.

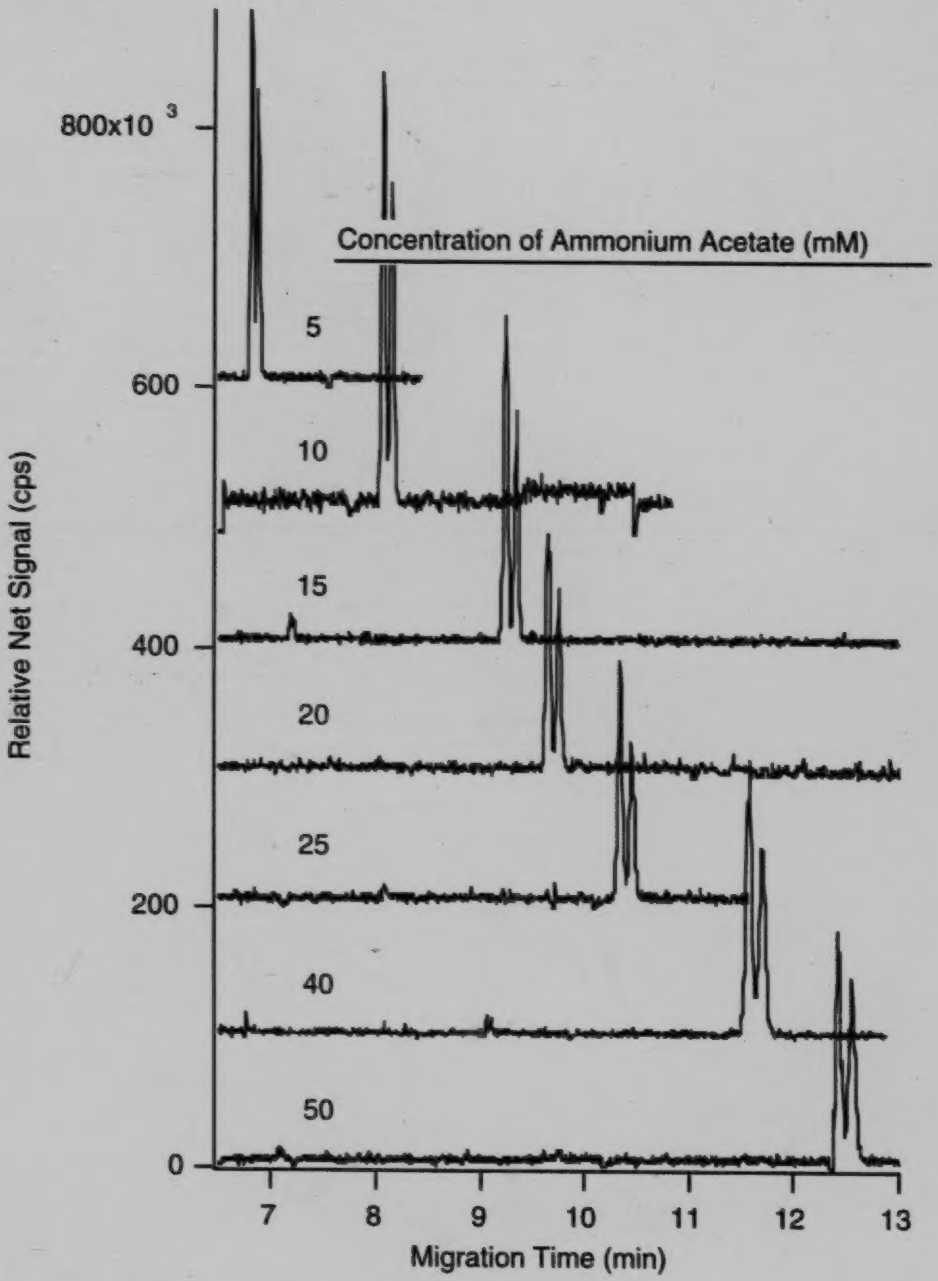
2.3.2.2 Electrolyte

The composition of a CE buffer can have a dramatic effect on the electrospray process and resulting signal. Non-volatile buffer systems often present background noise difficulties due to a suppression of analyte signal. Ammonium acetate is very volatile, and therefore an excellent electrolyte to use in buffer systems where electrospray is used. Figure 2.7 shows electropherograms where the 4–hydroxytamoxifen isomer standards are separated using various concentrations of ammonium acetate in a mixture of 70% methanol and 30% acetonitrile.

Increasing the concentration of ammonium acetate increases migration time. When a concentration of 5 mM was used, the migration time for the isomers was about 6.5 minutes. When concentration increased ten times to 50 mM, migration time nearly doubled to 12.5 minutes. As previously discussed (Section 2.3.2.1), slower migration time analyses, such as 50 mM in Figure 2.7, give a better resolution than faster migration times, such as 5 mM. In this particular example though, all peaks of the 4–hydroxytamoxifen isomers were well resolved.

Initially, it was decided that a concentration of 20 mM ammonium acetate would provide a good separation and fast analysis time of tamoxifen metabolites. However, as the experiment grew to a larger scale, time became more precious, so eventually the electrolyte concentration was decreased to 5 mM to allow a faster analysis time. At 5 mM, separation between the tamoxifen metabolites was still sufficient. The urine analysis (see Chapter 3) was done with 20 mM ammonium acetate and the serum analysis (see Chapter 4) was done with a 5 mM concentration of ammonium acetate in solvent. Comparisons

Figure 2.7. Extracted ion electropherograms (m/z 388) of the E and Z isomers of 4–hydroxytamoxifen in various concentrations of ammonium acetate in 70% methanol / 30% acetonitrile.



between urine and serum samples used concentrations of 5 mM.

In Figure 2.7, the analysis using 50 mM ammonium acetate had the highest concentration of electrolyte and therefore the highest ionic strength of the nonaqueous media. Electroosmotic flow decreases as the ionic strength increases. This is because the zeta potential decreases as a result of double-layer compression. Note the relationship between ionic strength and the zeta potential (Equation 1.4). The 50 mM ammonium acetate buffer had the lowest relative zeta potential and therefore the slowest migration time (see Equations 1.1 and 1.3). Another consideration is that low capillary current values due to low electrolyte concentrations are comparable to those generated in the CE/ESI/MS interface. Comparable CE and ESI currents mean the sensitivity limits of the mass spectrometer are maximized and the stability of the CE/ESI/MS system is optimal (18,28).

2.3.2.3 Injection Time, Injection Voltage, and Running Voltage

Resolution and peak intensity of extracted ion electropherograms for the 4–hydroxytamoxifen isomers were the factors used to determine optimal injection time, injection voltage, and running voltage. It was determined that an injection time, injection voltage, and running voltage of 5 seconds, 10 kV, and 20 kV respectively, were adequate. The actual running voltage would be less than 20 kV however, since the ionspray voltage at the cathodic end of the capillary is applied at about 4 kV. Therefore, the actual running voltage would be 16 kV.

2.3.3 Method Validation

2.3.3.1 Migration Time Comparison

Migration time for metabolites between patients is reproducible. Table 2.3 shows a comparison of metabolite migration times for 47 urine samples. The variation in migration

Table 2.3. Metabolite migration times for 47 urine samples.

Compound	Average Time ± Standard Deviation (min)	Relative Standard Deviation
N–desmethyltamoxifen	7.50 + 0.29	3.9%
tamoxifen	9.77 + 0.43	4.4%
4–hydroxytamoxifen	9.86 + 0.45	4.6%
tamoxifen-N-oxide	11.56 + 0.53	4.6%
3,4–dihydroxytamoxifen	11.82 + 0.72	6.1%

time shown here is normal for CE. The variation that does exist can be attributed mainly to the different lengths of capillary used (i.e. 60 cm was not the exact length; it was approximately 60 cm +/- 1 cm). Regardless, the absolute migration time reproducibility is not critical here. What is more important is the relative migration time, obtained by using an internal standard.

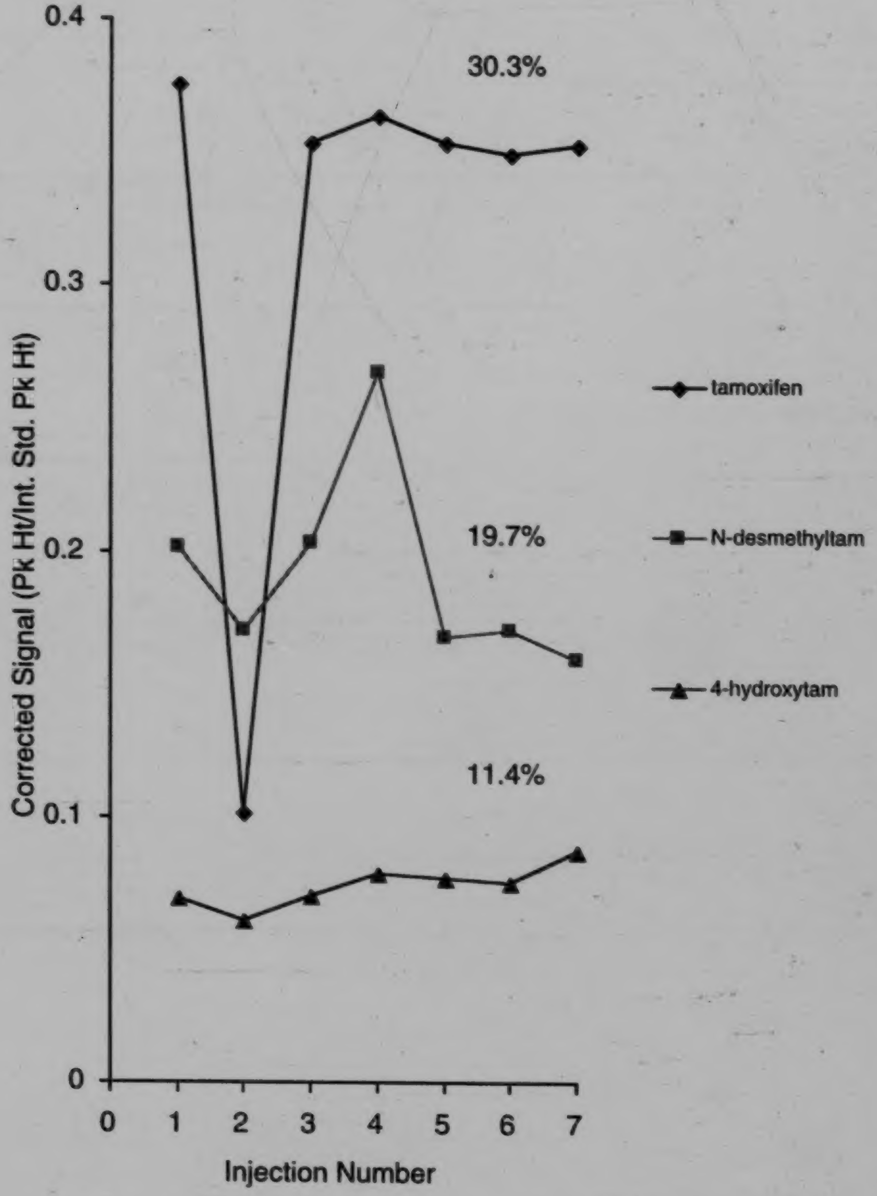
2.3.3.2 CE/ESI/MS Within-Day Precision: Analysis of Same Sample

Figure 2.8 shows results from seven runs of one urine sample extract intermittently analyzed over 13 hours for tamoxifen, N–desmethyltamoxifen, and 4–hydroxytamoxifen. Within the same day, CE/ESI/MS analyses gave repeatable results. Except for one of the seven analyses, tamoxifen always had the highest corrected signal (peak height less background divided by internal standard net signal) of the three compounds monitored. N–desmethyltamoxifen had the next highest signal, and 4–hydroxytamoxifen the least. The relative standard deviation (RSD) for the seven runs was 30.3% for tamoxifen, 19.7% for N–desmethyltamoxifen, and 11.4% for 4–hydroxytamoxifen. Using the Q-test, the second run for tamoxifen, which appears to be an anomaly, can be dropped from the data (the observed Q is 0.91, which is greater than the tabulated Q of 0.51). When this data point is taken out of the calculation for the RSD of tamoxifen, the RSD improves to 2.7%. Using the Q-test for N–desmethyltamoxifen, the fourth run, which also appears to be somewhat of an anomaly, can be discarded (the observed Q is 0.60, which is greater than the tabulated Q of 0.51). When this data point is taken out of the calculation for the RSD of N–desmethyltamoxifen, the RSD improves to 10.4%.

2.3.3.3 Absolute Concentration Determination of Metabolites in Patient Samples

The linear dynamic range in electrospray mass spectrometry is typically three or four orders of magnitude with an upper limit at 10^{-5}M. This upper limit is due to competition of ions for insufficient charge on droplets. When the analyte is less than 10^{-5}M

Figure 2.8. CE/ESI/MS within-day precision: Signal changes from same urine sample (with RSD).



in reagent-grade methanol, impurities dominate current. Above this upper limit, analyte dominates current. Concentration dependence then becomes non-linear (29). To quantitate unknowns, standards must be analyzed within the linear dynamic range, and from that calibration curves can be constructed. Two examples are shown in Figures 2.9 and 2.10. Absolute concentrations in six patients, analyzed over two days, have been determined from these calibration curves. Metabolic profiles of all six patients are shown in Figure 2.11.

In Figures 2.9 and 2.10, net signal (peak height less background signal) increases linearly and proportionally as concentration increases within the range analyzed, 0.005 μg/mL to 0.1 μg/mL. Tamoxifen, N–desmethyltamoxifen, and 4–hydroxytamoxifen standards were used to construct these calibration curves. Correlation coefficients were as low as 0.967 and as high as 0.999. These values are close to 1, indicating that net signal has a dependant relationship to concentration.

In Figure 2.11, metabolite absolute concentrations for these patients varied. From these six patients, the highest concentration for tamoxifen was 0.44 μg/mL, for N–desmethyltamoxifen it was 0.07 μg/mL, and for 4–hydroxytamoxifen it was 0.112 μg/mL. The lowest concentrations were undetectable for tamoxifen and N–desmethyltamoxifen, and for 4–hydroxytamoxifen it was 0.008 μg/mL. The means from the six patients were 0.183 ± 0.163 μg/mL for tamoxifen, 0.022 ± 0.031 μg/mL for N–desmethyltamoxifen, and 0.058 ± 0.043 μg/mL for 4–hydroxytamoxifen.

2.3.3.4 LC/MS Analysis

Using HPLC (PE 200 AutoSampler) with ESI/MS (PE Sciex API 3000), the following were analyzed: standards (tamoxifen, N–desmethyltamoxifen, 4–hydroxytamoxifen, tamoxifen–N–oxide, Metabolite Y, Metabolite E), two different patient's urine samples, and three different patient's serum samples. The purpose of this analysis was to further validate metabolite peak identification by using a different analytical

Figure 2.9. Calibration curve of tamoxifen and other metabolite standards: Day 1.

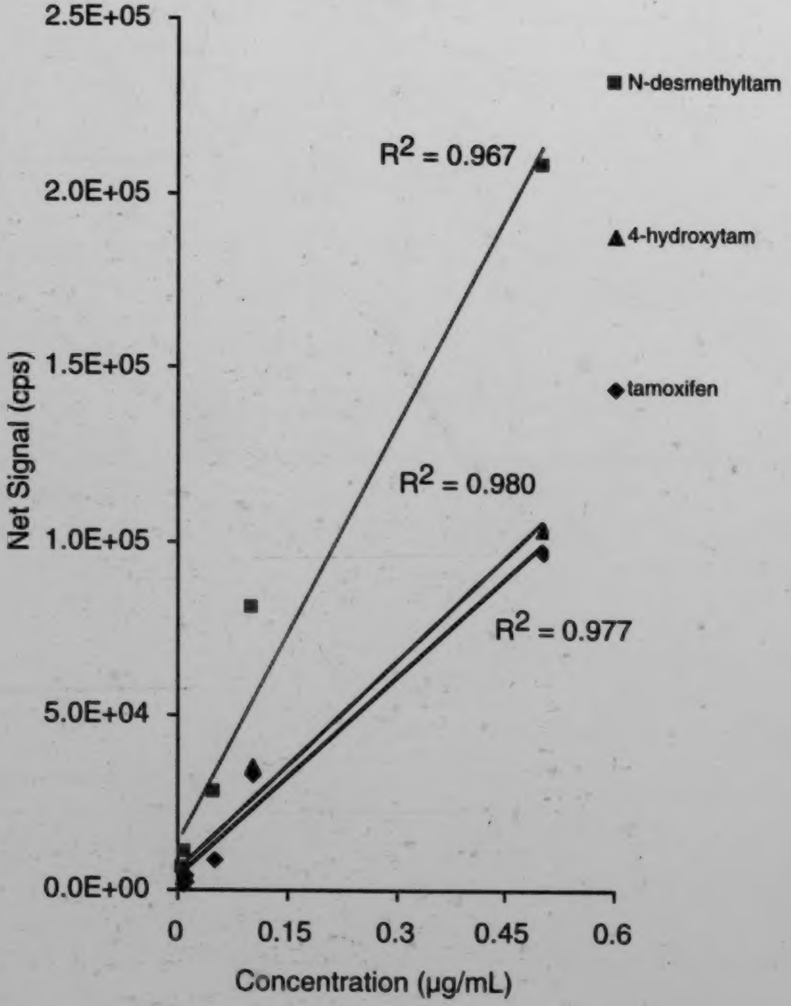


Figure 2.10. Calibration curve of tamoxifen and other metabolite standards: Day 2.

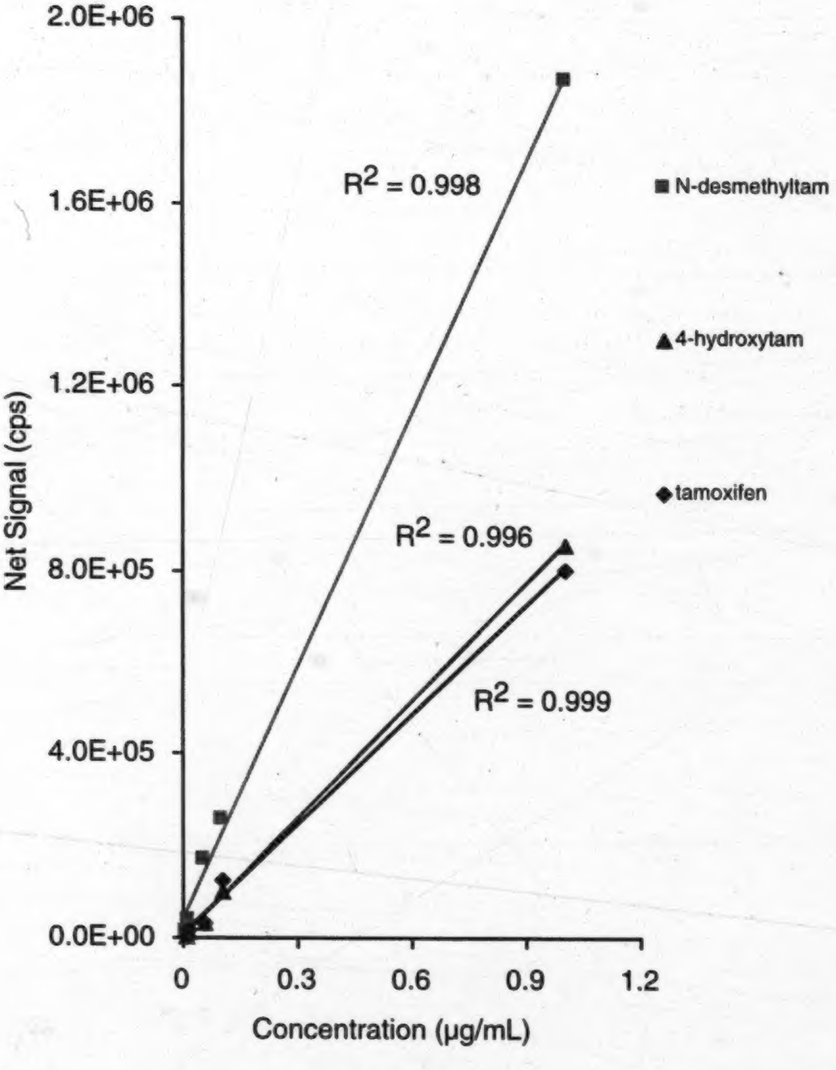
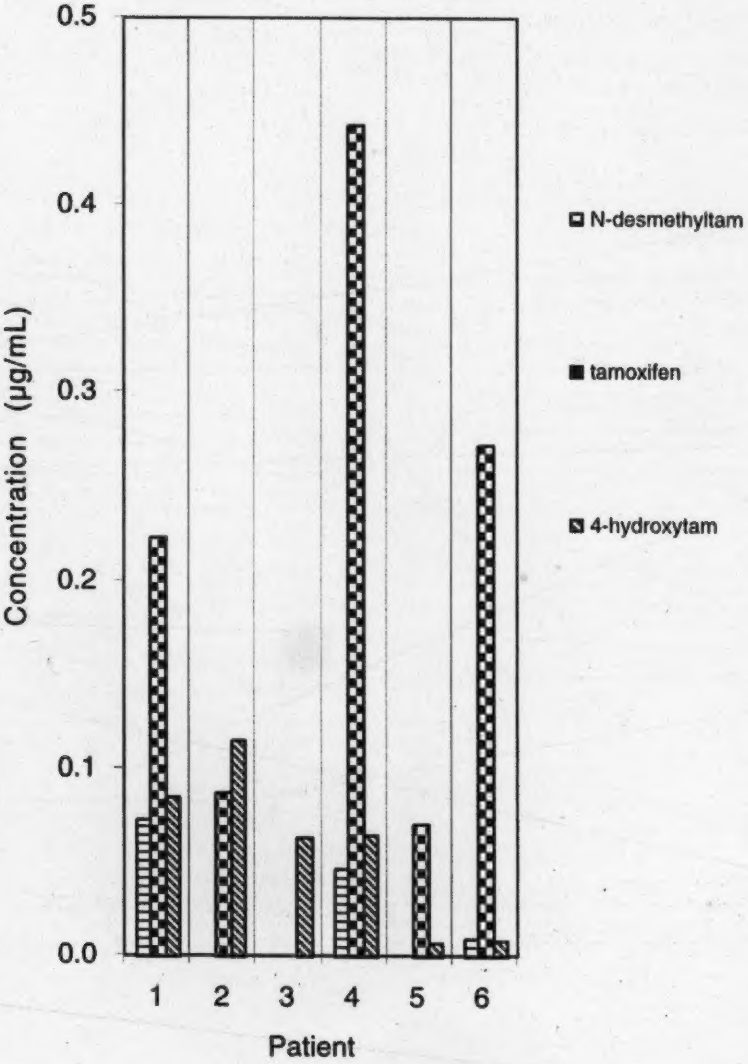


Figure 2.11. Metabolic profiles of six patients with concentrations determined from calibration curves of standards.



method. One concern with CE/ESI/MS analysis is whether some metabolites, because they are neutral, will migrate with other neutral compounds in the bulk electroosmotic flow, potentially interfering with the detection of the metabolite of interest. Analysis by LC/ESI/MS would alleviate this concern. Tamoxifen, N–desmethyltamoxifen, 4–hydroxytamoxifen, and tamoxifen–N–oxide peaks were identified in urine and serum patient samples. Metabolite Y was identified in serum but not urine. Metabolite E standard had no peaks and thus no identification of Metabolite E in urine or serum could be made. The strongest peak signals in urine and serum were for 4–hydroxytamoxifen and N–desmethyltamoxifen, respectively. Figures 2.12 and 2.13 show typical examples of how sample peaks were identified with standards in urine and serum, respectively.

In Figure 2.12, two metabolite isomers, 4-hydroxytamoxifen and tamoxifen–N–oxide, are identified from two peaks in an extracted ion chromatogram (m/z 388). The extracted ion chromatogram of m/z 388 for the patient's urine sample shows major peaks at 14.7, 15.0, and 16.3 minutes. 4–Hydroxytamoxifen standard produces a peak at 14.7 minutes. Tamoxifen–N–oxide standard produces a peak at 16.3 minutes. The peaks in the sample with similar migration times to those of the standards were identified as the respective metabolites. In the extracted ion chromatogram of m/z 388 for the blank sample, methanol, there were no peaks at the same migration times as the identified metabolites in the urine sample. The identity of extra peaks that are shown are unknown.

Figure 2.13 shows chromatograms from a patient's serum sample, a standard, and a blank sample. Both the total and extracted (m/z 358) ion chromatograms of the patient's serum sample are shown. The major peak, at 16.5 minutes, in the extracted ion chromatogram, has the same migration time as the peak in the extracted ion chromatogram for N–desmethyltamoxifen standard, and is therefore identified as the same metabolite. There are no peaks in the extracted ion chromatogram for blank methanol sample at 16.5

Figure 2.12. LC/MS metabolite identification. From top to bottom are extracted ion chromatograms (m/z 388) of 1) a patient's urine sample, 2) 4-hydroxytamoxifen standard, 3) tamoxifen–N–oxide standard, and 4) blank methanol sample.

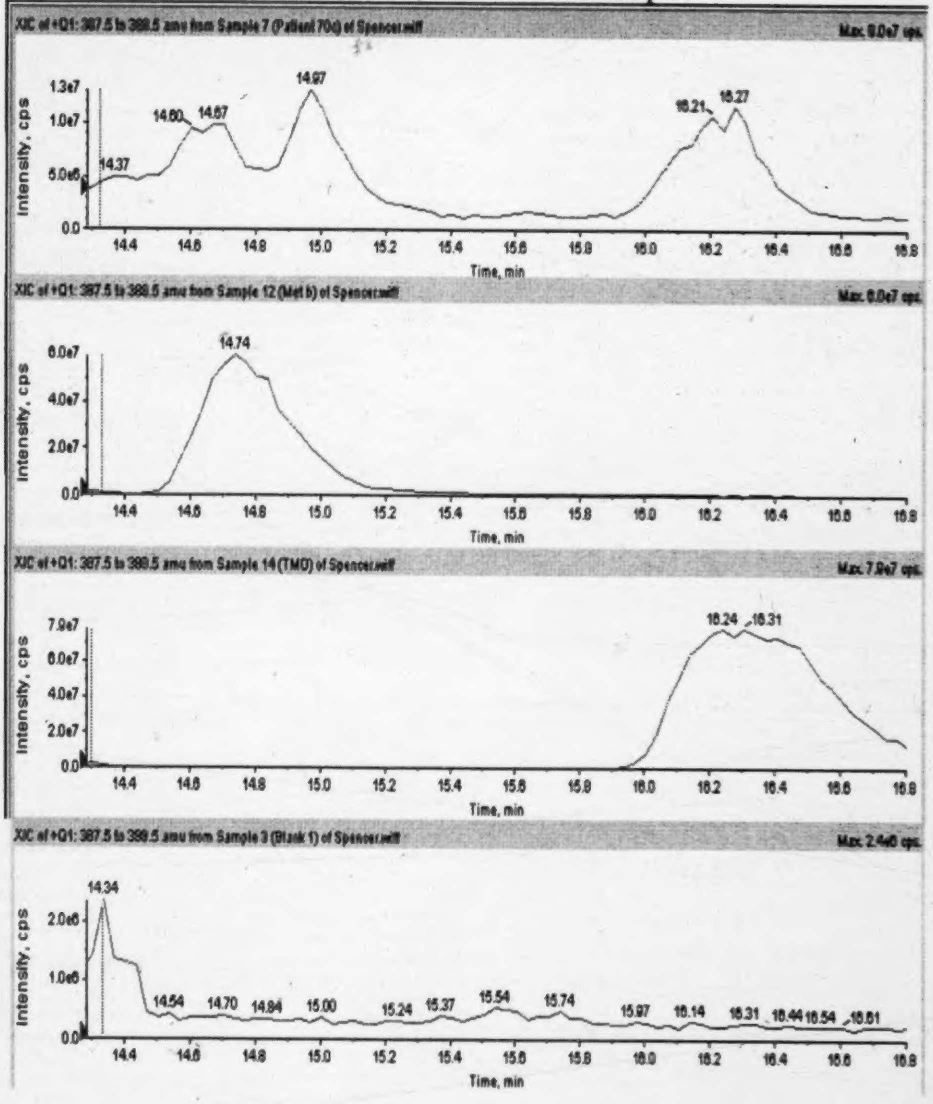
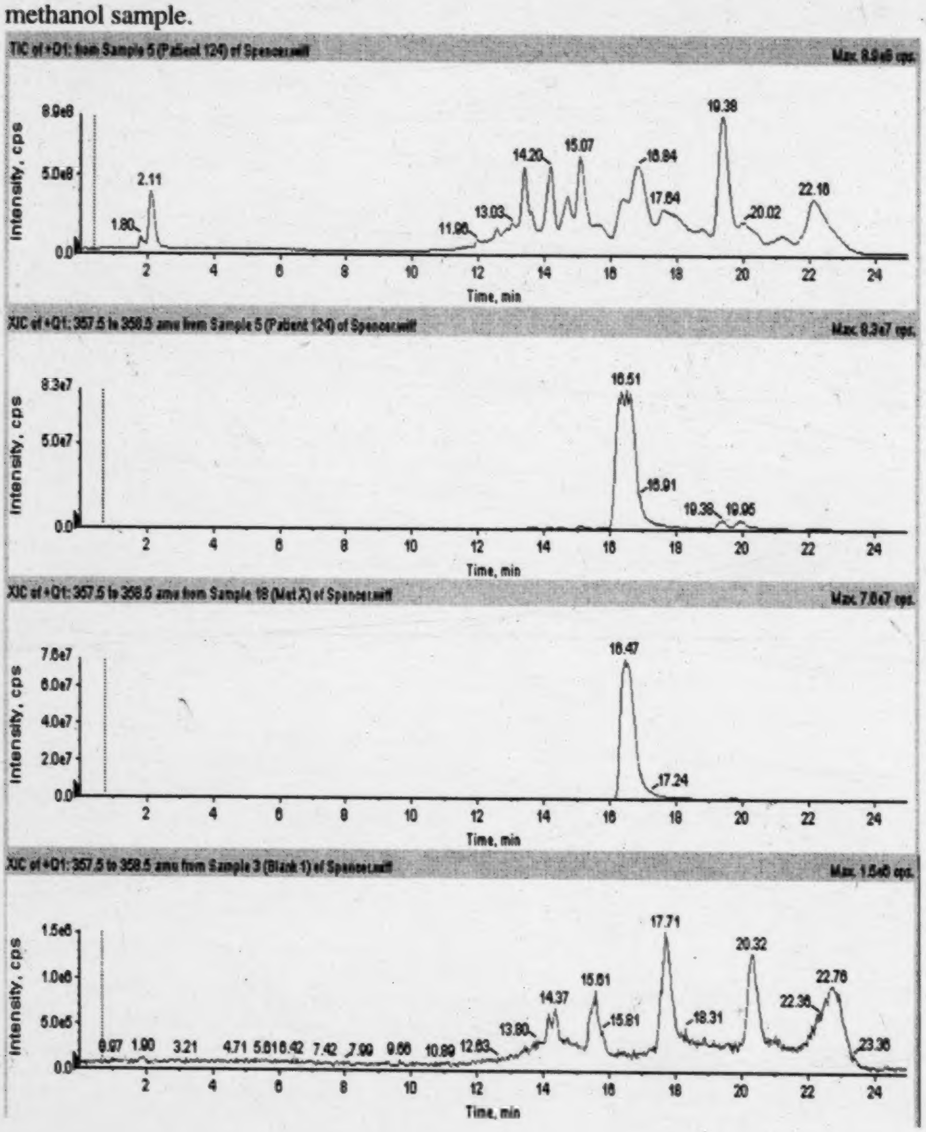


Figure 2.13. LC/MS metabolite identification. Top pane is 1) total ion chromatogram of a patient's serum sample. The following panes, in order, are extracted ion chromatograms (m/z 358) of 2) same serum sample, 3) N–desmethyltamoxifen standard, and 4) blank methanol sample.



minutes. The extra peaks in this blank sample are unknown. While some of the peaks in the total ion chromatogram of the patient's serum sample are from tamoxifen metabolites, the identity of most of these peaks remains unknown. These extra peaks may be residual from other experiments, since the HPLC column had been well used from other experiments.

2.3.3.5 Comparison to Previous Study

In the introduction, it was noted that nonaqueous CE/ESI/MS has already been used for the analysis of tamoxifen and metabolites (16). This study analyzed tamoxifen and its metabolites formed after incubation of tamoxifen with mouse hepatocytes. Besides this difference from the one here (Chapter 2), a surfactant was used in separation (the separation buffer was 2.5 mM ammonium acetate, 50 mM acetic acid, and 7 mM SDS in methanol). Surfactants can cause fouling of the ion source and analyte signals can be suppressed (17). Comparing this study to the one here, a detection limit, defined as S/N = 3, for N–desmethyltamoxifen was 10 μg/mL or 28 pmol/μL, whereas the detection limit for N–desmethyltamoxifen, using the first pane in Figure 2.2, was 7 ng/mL or 20 fmol/μL, an improvement of nearly three orders of magnitude. Resolution between peaks was sufficient, but migration times ranged from 35 to 56 minutes, which are about three to five times greater than those here (Table 2.3). Longer migration times are expected with a longer capillary (Equation 1.1), but the capillary was only 1.67 times longer than that used here (other experimental conditions in this study are: capillary was 100 μm i.d. x 100 cm in length, the running voltage was 25 kV, and the electrospray voltage was 2.48 kV).

2.3.4 Other Experimental Considerations

2.3.4.1 Stability of Samples

Tamoxifen is sensitive to UV light. The photolysis products of (Z)–tamoxifen are (E)–tamoxifen and other phenanthrenes formed by cyclization of both isomers (30). Temperature also has an effect on tamoxifen and its metabolites. In one experiment, the affinity of tamoxifen and 4–hydroxytamoxifen to the estrogen receptor in rat uterus decreased and increased respectively as temperature increased (31). In another experiment, after 2 days in a culture at 37°C, 20% of (Z)–4–hydroxytamoxifen isomerized to (E)–4–hydroxytamoxifen (32). Tamoxifen isomerization is a potential problem since the isomers have opposite biological effects. The Z isomer of tamoxifen, the form administered to the patient, is an antagonist, while the E isomer is an agonist.

2.3.4.1.1 Light and Temperature Effects on Same Day

Figures 2.14 through 2.16 demonstrate the effects of light and temperature within one day on patient samples. Tamoxifen, N–desmethyltamoxifen, and 4–hydroxytamoxifen were analyzed in each sample.

In Figure 2.14, no tamoxifen and N–desmethyltamoxifen were found in this particular patient's sample, while only trace amounts of 4–hydroxytamoxifen were found. This was the case even for the first analysis, in which the sample had not yet been exposed to light or room temperature. It is therefore inconclusive whether light and room temperature have a noticeable effect on a sample within the same day.

Figure 2.15 shows that light has little or no effect on the stability of a sample extract within a 12 hour period. Tamoxifen, N–desmethyltamoxifen, and 4–hydroxytamoxifen

Figure 2.14. Sample stability: Changes in sample at room temperature and exposed to light within same day.

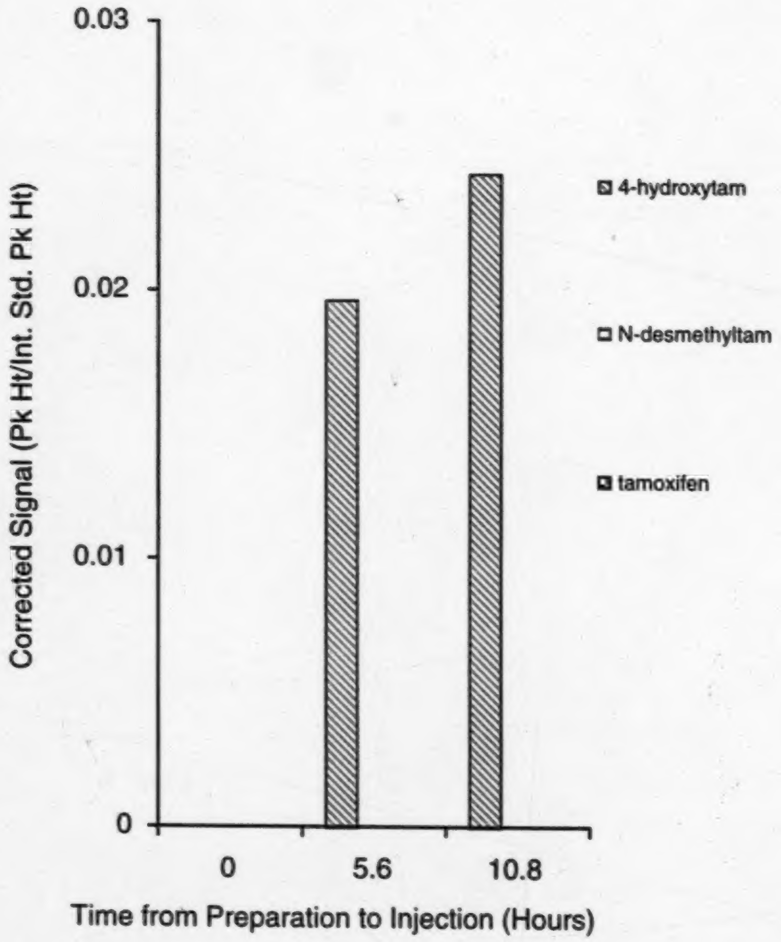
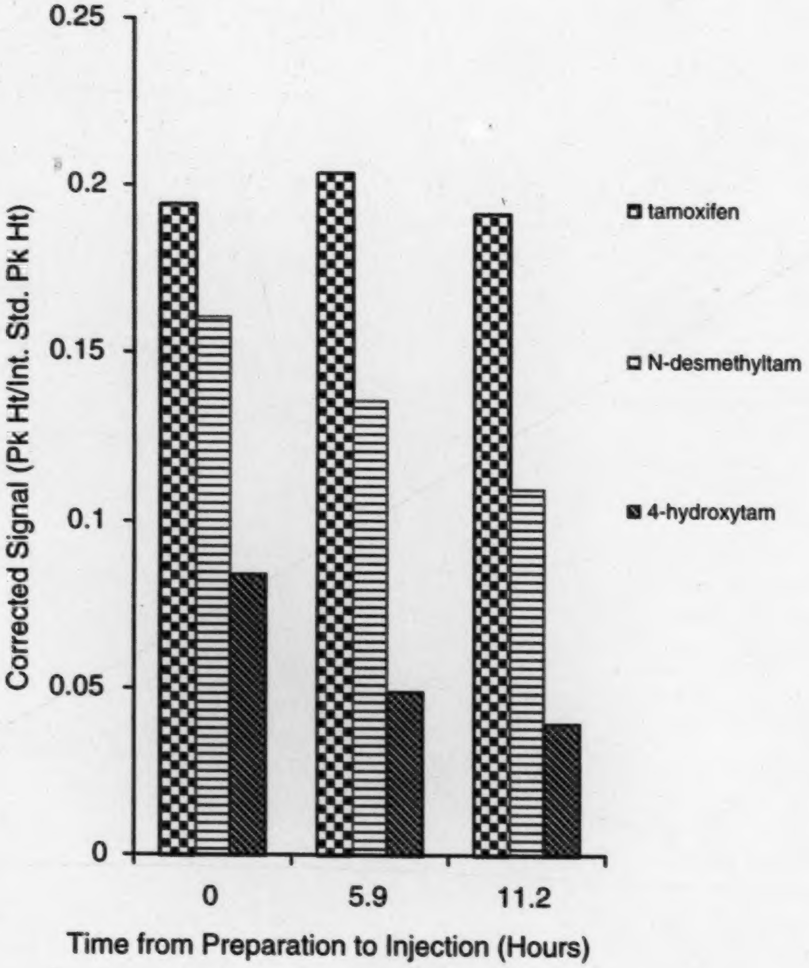


Figure 2.15. Sample stability: Changes in sample in ice and exposed to light within same day.



were found in this particular sample. The RSD for the corrected signal was 3% for tamoxifen, 19% for N–desmethyltamoxifen, and 40% for 4–hydroxytamoxifen. Except for 4–hydroxytamoxifen, these values are less than the RSD values determined for the CE/ESI/MS repeatability experiment (Section 2.3.2.2).

A sample extract is likely to remain stable within a 12 hour period if kept in the refrigerator. In Figure 2.16, tamoxifen, N–desmethyltamoxifen, and 4–hydroxytamoxifen were found in this particular patient's sample. The RSD for the corrected signal was 14% for tamoxifen, 13% for N–desmethyltamoxifen, and 37% for 4–hydroxytamoxifen. Except for 4–hydroxytamoxifen, these values are less than the RSD values determined for the CE/ESI/MS repeatability experiment (Section 2.3.2.2).

Notwithstanding these results, all precautions to avoid light and higher temperatures (> 4°C) were taken when analyzing patient samples. Samples were analyzed shortly (within 30 minutes) after they were redissolved in methanol and afterwards stored in the refrigerator in UV–protected vials.

2.3.4.1.2 Analysis of Same Sample over Longer Period

When analyzing the same sample over a longer period (more than one day), two issues arise: First, is the sample stable over the entire period? Second, is the instrumentation for analysis reproducible over that time period? A typical example of how one sample's measured metabolites changed in two weeks is shown in Figure 2.17. Measured metabolite signals changed very little for the same sample over a two-week period. In the example shown in Figure 2.17, the net signal RSD between the two time periods ranged from 3% for 4–hydroxy–N–desmethyltamoxifen to 25% for 4–hydroxytamoxifen. The longest time-period examined for one patient's sample was just over one month.

Figure 2.16. Sample stability: Changes in sample at 4°C and no light exposure within same day.

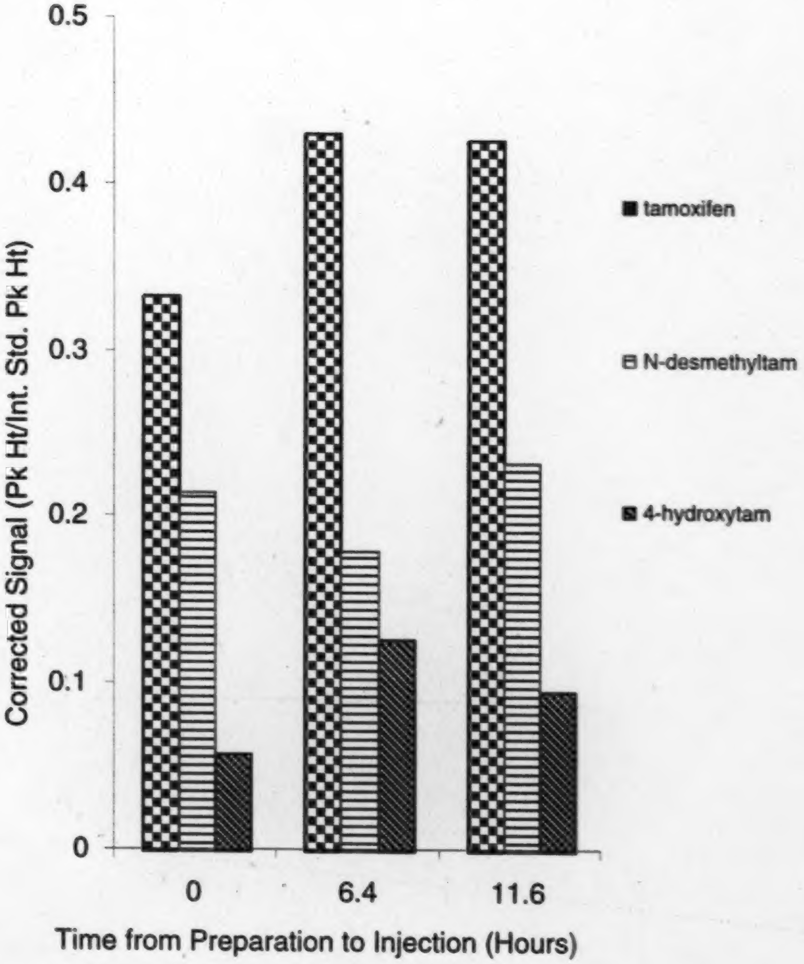
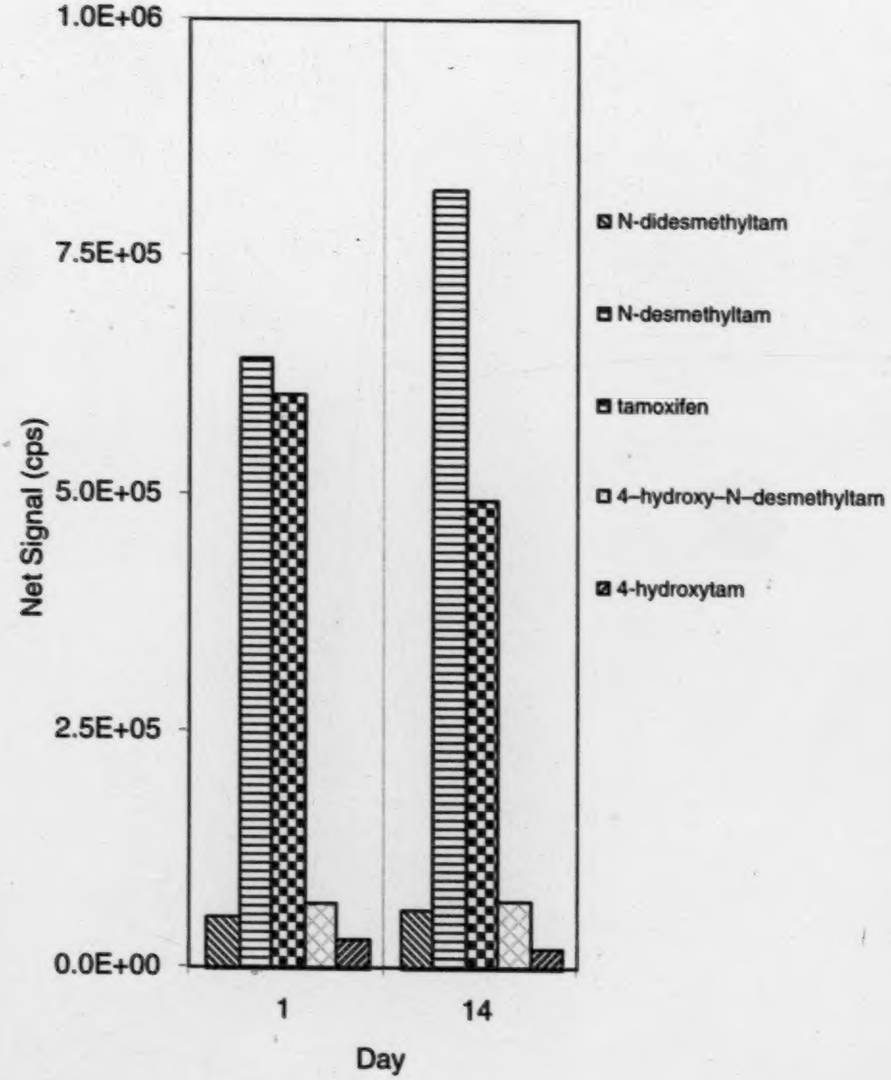


Figure 2.17. Sample stability and reproducibility: Signal changes from same serum sample over two weeks.



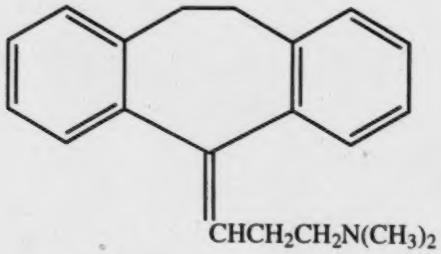
2.3.4.2 Relative Comparisons

The major focus in these experiments was to make comparisons of metabolite levels between patients. In order to make such comparisons, corrections in the net signal for metabolite peaks needed to be made. These corrections included the use of an internal standard.

2.3.4.2.1 Internal Standards

Precision in quantitative analysis increases when an internal standard is used. Uncertainty introduced by the sample or from the instrument lessens. For separations, the internal standard should have a similar migration time to other metabolites and yet its peak should not overlap other metabolite peaks. For mass spectrometry detection, internal standards are often isotopes of the analyte. In considering what the internal standard should be, an isotope of one of the metabolites was not used so that bias would not be introduced with the internal standard being similar to one metabolite more than the others would. The ideal internal standard for this experiment would have a similar structure and molecular weight to all of the detected metabolites. Finding such an internal standard would be difficult partly because similarity in structure to all the metabolites would likely mean it would be a metabolite itself. A number of antidepressant drugs (amitriptyline, clomipramine, desipramine, imipramine, and nortriptyline) were analyzed to determine an appropriate internal standard. These compounds were similar in structure and molecular weight to tamoxifen and its metabolites. Initially, amitriptyline was used as the internal standard. Its structure is shown in Figure 2.18.

Figure 2.18. Structure of amitriptyline.


CHCH₂CH₂N(CH₃)₂

Amitriptyline separated well from tamoxifen metabolites. Amitriptyline, like all of the tamoxifen metabolites listed in Chapter 1 (Table 1.3) is tricyclic. It has two phenyl rings and has a dimethyl amino side chain similar to the dimethyl amino side chain on tamoxifen. Amitriptyline has a molecular weight of 277.39 g mol^{-1}.

For the analysis of urine samples (Chapter 3), amitriptyline was added after the extraction process but before the CE/ESI/MS analysis. As it became clear that this experiment would become more large-scale in the number of patient samples analyzed, an internal standard needed to be added before the extraction process, not just before CE/ESI/MS analysis. Therefore, for the serum analysis (Chapter 4), a new internal standard, imipramine, was added to the patient's sample before extraction. Its structure is shown in Figure 2.19.

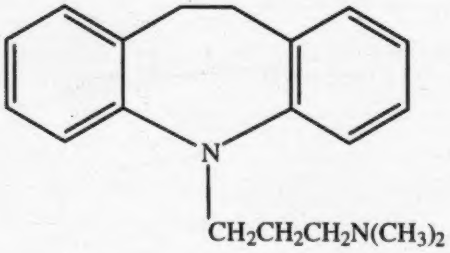
Amitriptyline continued to be added to samples after extraction but before CE/ESI/MS analysis for serum analysis. Similar to amitriptyline, imipramine is easily identifiable and separates well in CE from other tamoxifen metabolites. Imipramine is also tricyclic with two phenyl rings and has a dimethyl amino side chain. It has a molecular weight of 280.40 g mol^{-1}.

2.4 Conclusions

A method for the analysis of tamoxifen and its metabolites using nonaqueous CE/ESI/MS was developed. CE separation was optimized with respect to the solvent and electrolyte of the buffer, injection time and voltage, and the running voltage. Metabolites were identified in patient's urine and serum samples.

The method was validated with respect to within-day precision by repetitively analyzing the same sample on one day, and to between-day precision by comparing migration times between many patients analyzed on different days and analyzing the same

Figure 2.19. Structure of imipramine.


N
CH₂CH₂CH₂N(CH₃)₂

patient's extract on different days. Calibration curves were constructed from the analysis of standards and absolute concentrations were determined in a few patients. LC/ESI/MS was used as a comparison of analytical methods. The stability of samples within one day and over a larger period and relative comparisons between patients was also considered.

Tamoxifen and its metabolites in urine and serum samples can be accurately and precisely measured using nonaqueous CE/ESI/MS.

2.5 References

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Chapter 3

Analysis of Tamoxifen and Its Metabolites in Urine from Breast Cancer Patients

3.1 Introduction

Tamoxifen and its metabolites in urine have been analyzed in the past (1-4). Most of these analyses were performed by liquid chromatography (LC) with mass spectrometry (MS) or ultraviolet (UV) detection. Tamoxifen metabolites found in urine have not yet been analyzed, according to literature searches, by aqueous or nonaqueous capillary electrophoresis with electrospray mass spectrometry (CE/ESI/MS). The analysis of tamoxifen metabolites found in urine is difficult because a small proportion of metabolites, approximately 20%, is excreted into urine (5). Tamoxifen and its metabolites are excreted primarily by the biliary route. One study showed that the highest proportion of tamoxifen and 4–hydroxytamoxifen was found in feces (5). Although 20% is not a large fraction, interpatient comparisons of urinary metabolite profiles that indicate the preferential metabolic activities of individuals can still be performed if extraction procedures are well planned to obtain a high recovery and analytical instrumentation provides sensitive measurements. Despite this analytical challenge in analyzing urine, the advantages are that samples are easily obtained and non-invasive to the patient. Typically, the major metabolite found in urine is 4–hydroxytamoxifen (5).

Tamoxifen's primary mechanism of action is competitive inhibition of the estrogen receptor-alpha, thereby inhibiting growth of malignant breast cells (6-9). Tamoxifen is used to control advanced or metastatic breast cancer (stage IV disease) (10), where it benefits approximately half of patients by inducing tumor shrinkage or stabilization for at least six months. For patients with locally confined breast cancers (stage I, II, or III diseases), tamoxifen administration for five years reduces the risk of recurrence of breast cancer both locally and systemically and improves overall survival rates (11). For women at high risk of developing breast cancer, five years of tamoxifen administration reduces the incidence of breast cancer by approximately 50% (12). However, despite these proven benefits for treated populations, the use of tamoxifen is limited by the eventual development of acquired resistance in many patients. Many explanations for this therapeutic failure have been proposed, including interpatient and intratumoral variations in tamoxifen metabolism, but this has not yet been definitively established (13-19).

The purposes of this study of tamoxifen metabolism in urine are (i) to optimize and validate nonaqueous CE/ESI/MS as a qualitative and quantitative technique for clinical analysis of tamoxifen and its metabolites, (ii) to investigate how tamoxifen is metabolized in humans and confirm the hypothesis of differential metabolism in individual patients, and (iii) to explore relationships between patient urine tamoxifen metabolic profiles and clinical characteristics.

3.2 Experimental

3.2.1 Chemicals

(Z)–Tamoxifen was purchased from Sigma (St. Louis, MO, U.S.A.). (Z)–4–hydroxytamoxifen was purchased from Research Biochemical International (Natik, MA, U.S.A.). (Z)–N-desmethyltamoxifen was obtained from Sciex (Concord, ON). Tamoxifen–N–oxide was synthesized following a procedure outlined in the literature (20). 3,4–Dihydroxytamoxifen standard was not obtained, but was identified in patient samples by in-source CID (Figure 2.4). HPLC–grade methanol and acetonitrile were purchased from Caledon (Georgetown, ON) and EM Science (Gibbstown, NJ, U.S.A.), respectively. Ammonium acetate was purchased from ACP Chemicals (Montreal, PQ). The CE running buffer was 20 mM ammonium acetate in methanol-acetonitrile (70:30).

3.2.2 Urine Samples

Forty-seven urine samples were obtained from patients taking 20 mg of tamoxifen (Nolvadex, Astra-Zeneca) daily by mouth. All patients had been diagnosed with invasive ductal carcinoma of the breast (stages I, II, III or IV) and were being followed at a comprehensive cancer clinic (Cross Cancer Institute, Edmonton, AB). All patients had estrogen receptor positive disease determined by immunohistochemistry using antibody H226. Samples were stored at –20°C until analysis. All patients gave informed consent and the Cross Cancer Institute Research Ethics Committee approved the research protocol. A blank sample was also taken from a healthy volunteer not taking tamoxifen for analysis.

3.2.3 Extraction of Metabolites

Similar to the procedures that Poon et al. (1) reported, tamoxifen metabolites were extracted from 10 mL of urine with hexane containing 2% butanol (2 x 10 mL). The organic layers were combined and evaporated using a roto-evaporator. The residues were transferred into a glass vial and dried under argon gas. The dry residues were kept at 4°C. Samples were redissolved in 100 µL methanol with the internal standard, amitriptyline, just before CE injection.

3.2.4 Capillary Electrophoresis

An in-house constructed CE system was used for separations. Untreated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, U.S.A.) with 60 cm x 50 µm i.d. x 150 µm o.d. dimensions were used for separations. Fresh running buffers were used to flush the capillary before and between runs. Electrokinetic injections at 10 kV for 5 s were used to introduce samples into the capillary. Separations were performed at 20 kV. The operating electrolyte was changed every 3 to 5 runs to avoid contamination and solvent evaporation. All runs were done at ambient temperature.

3.2.5 Electrospray Ionization Mass Spectrometry

A PE/Sciex API 100 LC single quadrupole mass spectrometer equipped with an ion spray source was used to analyze eluents. The CE column was inserted 0.5-1.0 mm inside the tip of the ESI electrode, and the electrode was 0.5-1.0 mm outside the nebulizer tube. A Harvard Apparatus low-pressure syringe infusion pump (South Natick, MA, U.S.A.)

was used to pump methanol, the coaxial sheath liquid. The flow rate was 2.0 µL/min through a capillary of 60 cm x 50 µm i.d. x 50 µm o.d. dimensions connected to the hold-down tee of the ion sprayer, and to the electrode tube surrounding the CE separation column. Positive ion mode was used for all measurements. Ion spray voltages ranged from 3500 to 4600 V, orifice voltages from 10 to 25 V, ring voltages from 150 to 260 V, nebulizer gas flow rates from 0.03 L/min (NEB 0) to 0.67 L/min (NEB 1), CEM voltages from 2600 to 3000 V, and the curtain gas flow from 0.95 L/min (CUR 8) to 1.08 L/min (CUR 9). A working pressure of 1.1 x 10^{-5} Torr was maintained in the analyzer chamber. During sample injections, the ionspray was set to 0 V and the nebulizer gas flow rate was set to 0.03 L/min (NEB 0). For data acquisition the mass to charge (m/z) range was 50-800 amu, step size was 1.0 amu, scan speed was 1.13 s, dwell time was 1.5 ms, and pause time was 3.0 ms. Electropherograms were acquired by a LC Tune software (PE Sciex, Concord, ON), and then processed by Multiview 1.2 software from Sciex. All assays were blinded with regard to the clinical characteristics of the patients.

3.2.6 Creatinine analysis

Although urine output volume varies substantially day-to-day, the amount of creatinine (the end product of creatine metabolism) excreted daily into urine remains relatively constant. Creatinine concentrations were determined by high-performance liquid chromatography separation with UV detection following the procedure of Achari *et al.* (21). Briefly, 100 µL of urine added to 500 µL mobile phase was pushed through a syringe cartridge (Supelco, ENVI-18). The eluant was then diluted by a factor of 10 in water.

15 µL of sample was injected (Waters 712 WISP) into an ODS(3) (250 mm x 4.6 mm) column. The mobile phase was 50 mM sodium acetate in 98% water / 2% acetonitrile at a pH of 6.5. The flow rate was 0.8 mL/min. The UV absorbance detector (Waters 484

Tunable Absorbance Detector) was set to a wavelength of 230 nm. Standards ranging from 10 to 2000 μg/mL were used to construct a calibration curve.

3.3 Results

3.3.1 Creatinine Analysis

A typical calibration curve is shown in Figure 3.1. In this example, concentrations ranging from 100 μg/mL to 2000 μg/mL were analyzed at least three times each. Peak areas were measured, and the data were fit with a straight line. R^2 was 0.992, showing a high dependence of the signal to the standard concentration.

Figure 3.2 shows a chromatogram of a 1000 μg/mL creatinine standard. A chromatogram of a typical urine patient sample is shown in Figure 3.3. The creatinine concentration of this particular patient, determined from a calibration curve, was 750 ± 74 μg/mL. Elution times for the patient sample peaks in Figure 3.3 were compared to the elution time of the creatinine standard peak in Figure 3.2. The creatinine peak usually had a migration time slightly less than 5 minutes. A dip about 3.5 minutes appeared in standard and sample chromatograms and another peak usually appeared at about 4 minutes in sample chromatograms. The identity of this dip and peak are unknown.

These results were used to correct for differences in urine volume between patients by dividing the metabolite signal (peak height minus background) by the creatinine concentration. Table 3.1 shows some statistics of the creatinine concentrations that were determined in 100 patient urine samples. Note the large variation in creatinine concentrations between patients. Such wide variation had a great effect on the corrected signal.

Figure 3.1. Calibration curve for creatinine standards.

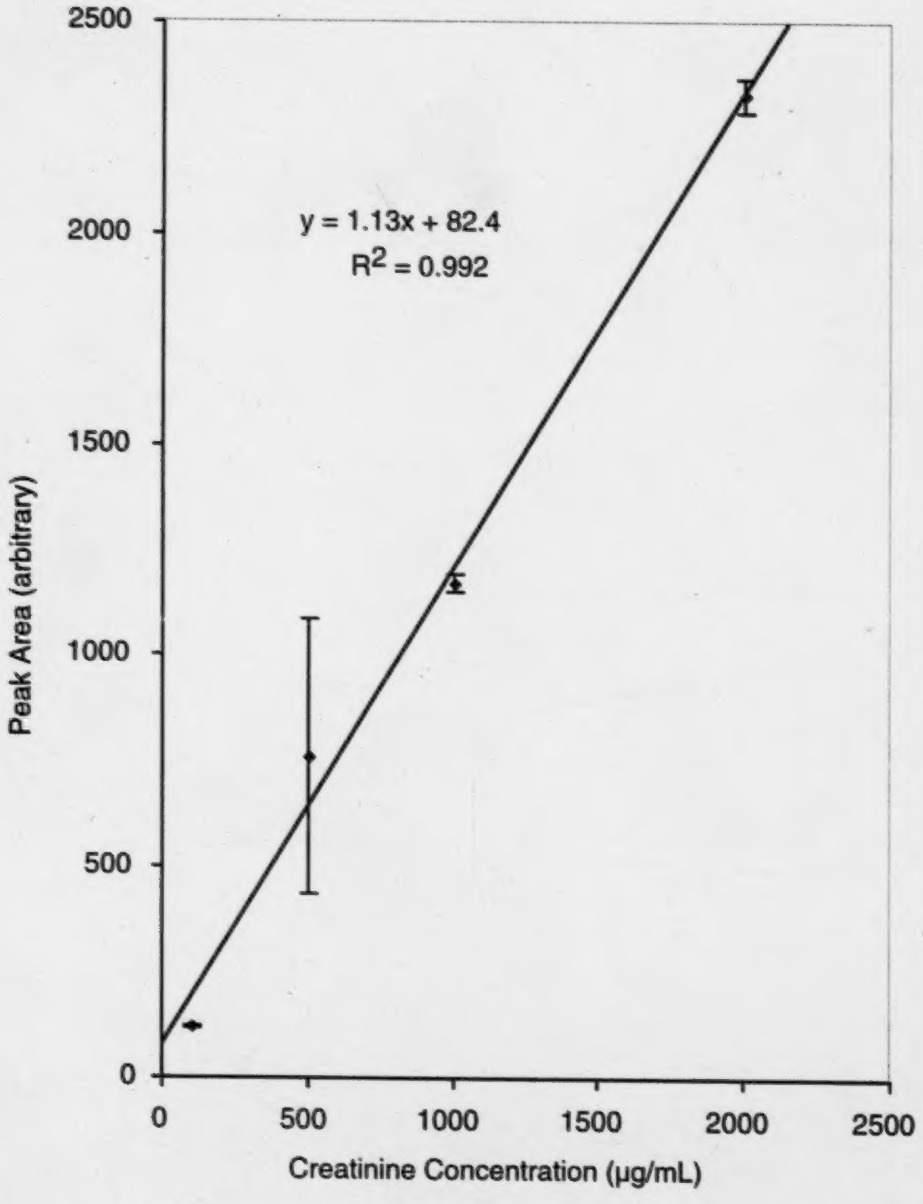


Figure 3.2. HPLC chromatogram of 1000 µg/mL creatinine standard.

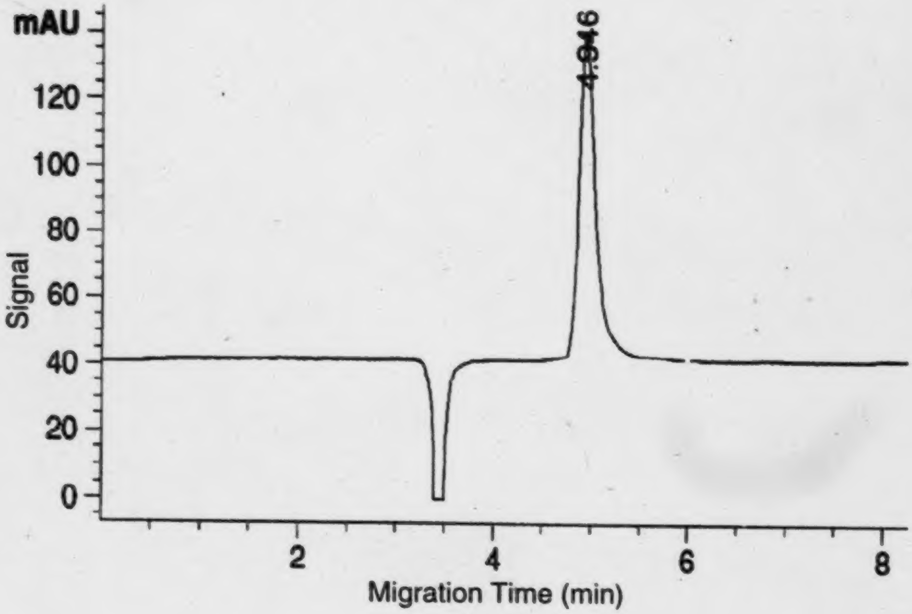


Figure 3.3. HPLC chromatogram of creatinine in urine patient sample.

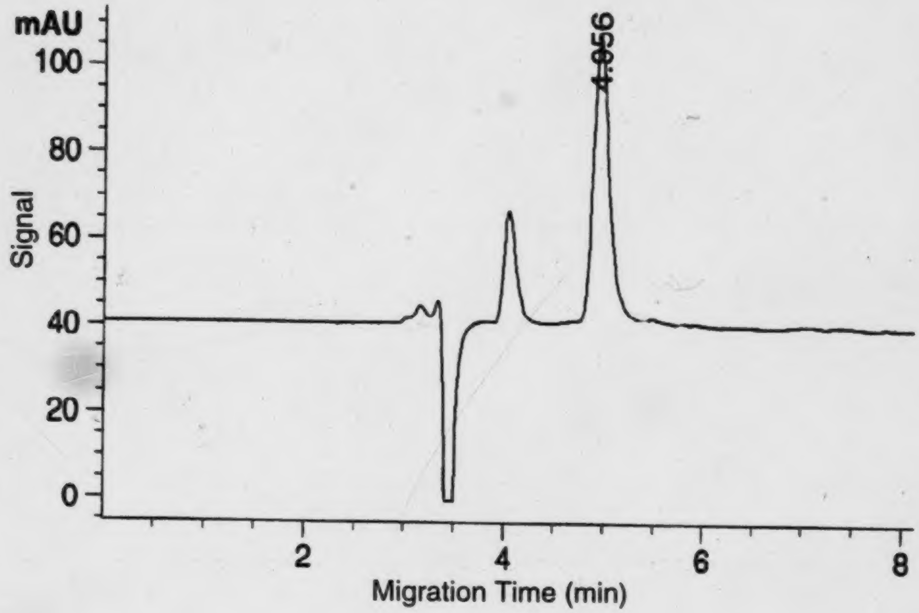


Table 3.1. Statistics for creatinine concentrations determined by HPLC in 100 patient urine samples (all numbers in μg/mL).

Average	**Standard Deviation**	**Median**	**Maximum**	**Minimum**
690	500	660	2100	10

3.3.2 Urine Extraction and Analysis Repeatability

This section is different from Section 2.3.2.2 because the repeatability for the entire urine sample analysis procedure – extraction and CE/ESI/MS analysis was determined. Section 2.3.2.2 focuses on the repeatability for the CE/ESI/MS analysis only. Here, three replicates of the same urine sample were extracted and analyzed separately on the same day. The profiles of metabolites were repeatable as shown in Figure 3.4. The extraction of tamoxifen metabolites in urine samples and analysis with nonaqueous CE/ESI/MS is repeatable. Figure 3.4 shows similar metabolite profiles from three replicate samples. The relative standard deviation (RSD) of peak heights for these urine samples were 11% for tamoxifen, 10% for N–desmethyltamoxifen, 54% for 4–hydroxytamoxifen, and 47% for 3,4–dihydroxytamoxifen. No tamoxifen–N–oxide was detected. Although the RSD values are not as low as desired for each metabolite, they demonstrate satisfactory variations in the process from sample extraction to instrument analysis.

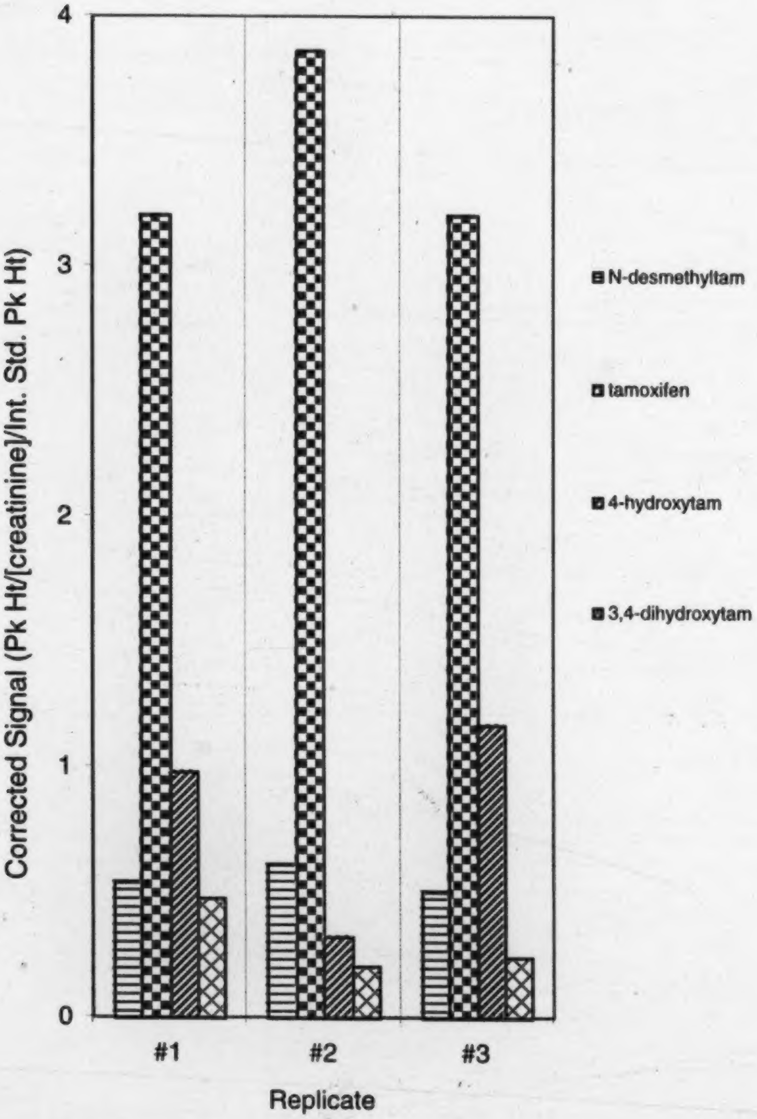
3.3.3 Changes in Patient Metabolic Profiles over Time

To determine if metabolic profiles vary within patients over time, two experiments were done. The first looked at metabolism within one day and the second examined metabolism over a two week period. Eight patients collected three urine samples on one day and repeated this again, two weeks later. In total, 48 samples were analyzed (see Appendix I).

3.3.3.1 Metabolism within the Same Day

Patients were instructed to collect three urine samples of approximately 100 mL each, with eight hour intervals between collections at, for example, 8 a.m., 4 p.m., and midnight. From each of these samples, five metabolites were analyzed: tamoxifen, N–desmethyltamoxifen, 4–hydroxytamoxifen, tamoxifen–N–oxide, and

Figure 3.4. Repeatability of the entire analytical procedure: Three replicates from the same urine sample analyzed on the same day.



3,4–dihydroxytamoxifen. Two examples of how a patient's urinary metabolic profile varies within one day are shown in Figures 3.5 and 3.6.

There is little variation in urinary metabolic profile for patients within the same day. Figure 3.5 shows the urinary metabolic profile for a stage I patient that had been taking tamoxifen for just over 7 months. Compared to other metabolites, 3,4–dihydroxytamoxifen was in greatest abundance, followed in order by tamoxifen, 4–hydroxytamoxifen, N–desmethyltamoxifen, and tamoxifen–N–oxide.

Figure 3.6 shows the urinary metabolic profile for a stage II patient that had been taking tamoxifen for just over 25 months. Tamoxifen was found in greatest abundance, followed in order by N–desmethyltamoxifen, 4–hydrdoxytamoxifen, 3,4–dihydroxytamoxifen, and tamoxifen–N–oxide.

From the eight patients, means and standard deviations for metabolites were calculated, with results being pooled and divided into groups. Each metabolite and the different times of day was a group (e.g. tamoxifen morning, tamoxifen afternoon, tamoxifen evening, N–desmethyltamoxifen morning, etc.). Paired t-tests were done on the three groups of each metabolite, comparing the time of day (e.g. tamoxifen morning and tamoxifen afternoon; tamoxifen morning and tamoxifen evening; tamoxifen afternoon and tamoxifen evening) to determine any significant differences. P-values of less than 0.05 would indicate a significant difference. The range of p-values was from 0.25 to 0.96. All p–values were well above 0.05. Two weeks later, patients repeated the same procedure, collecting their urine three times on one day. The same analysis was performed and the same result was obtained. P-values ranged from 0.15 to 0.76. No significant differences from these analyses are an indication that a patient's urinary metabolic profile does not significantly alter within the same day.

Figure 3.5. Urinary metabolic profile variation within the same day (first example).

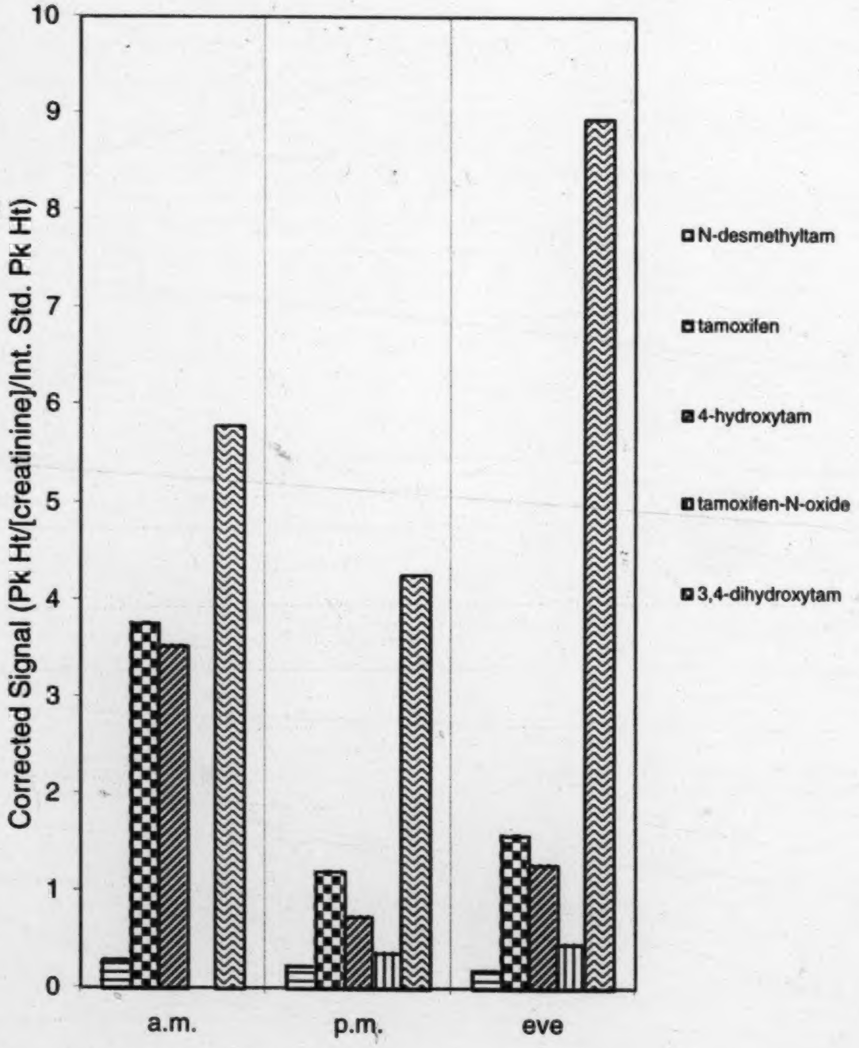
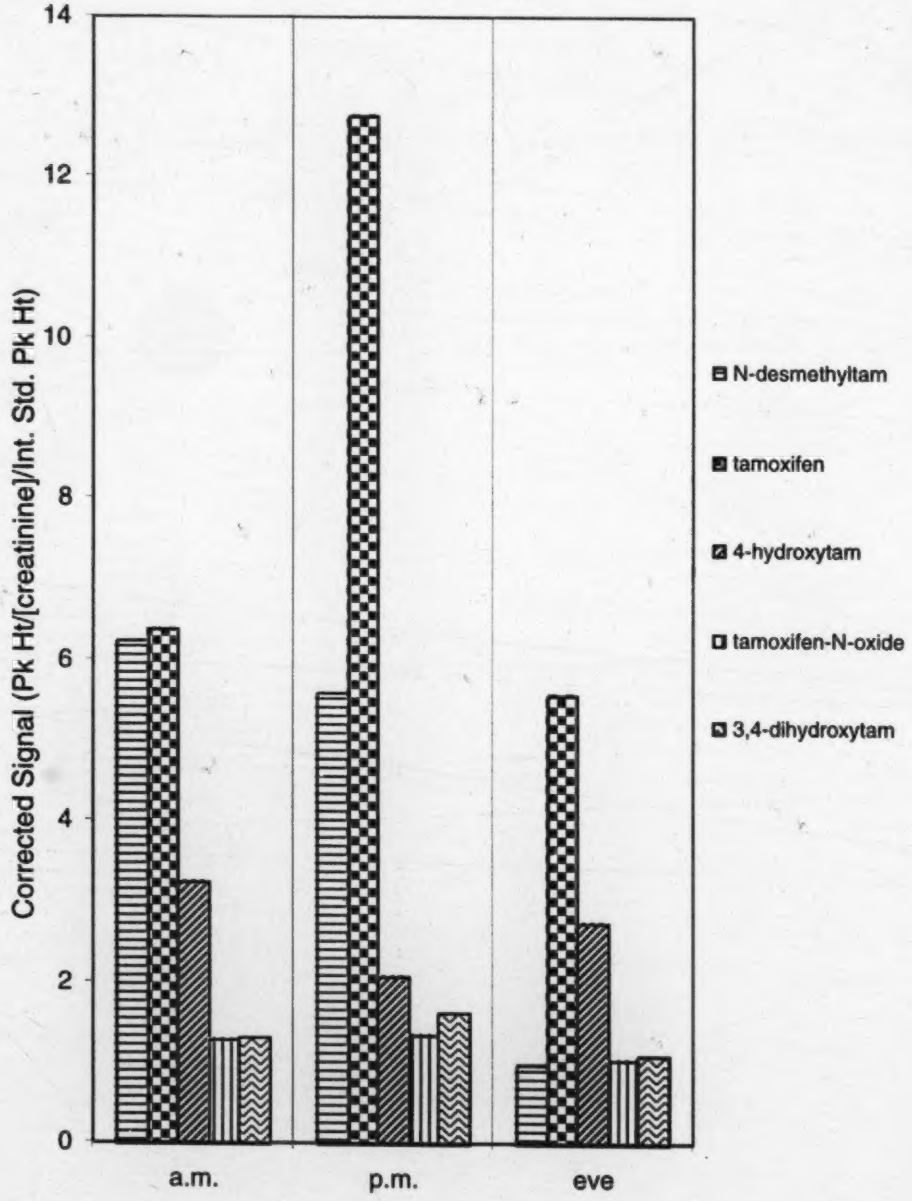


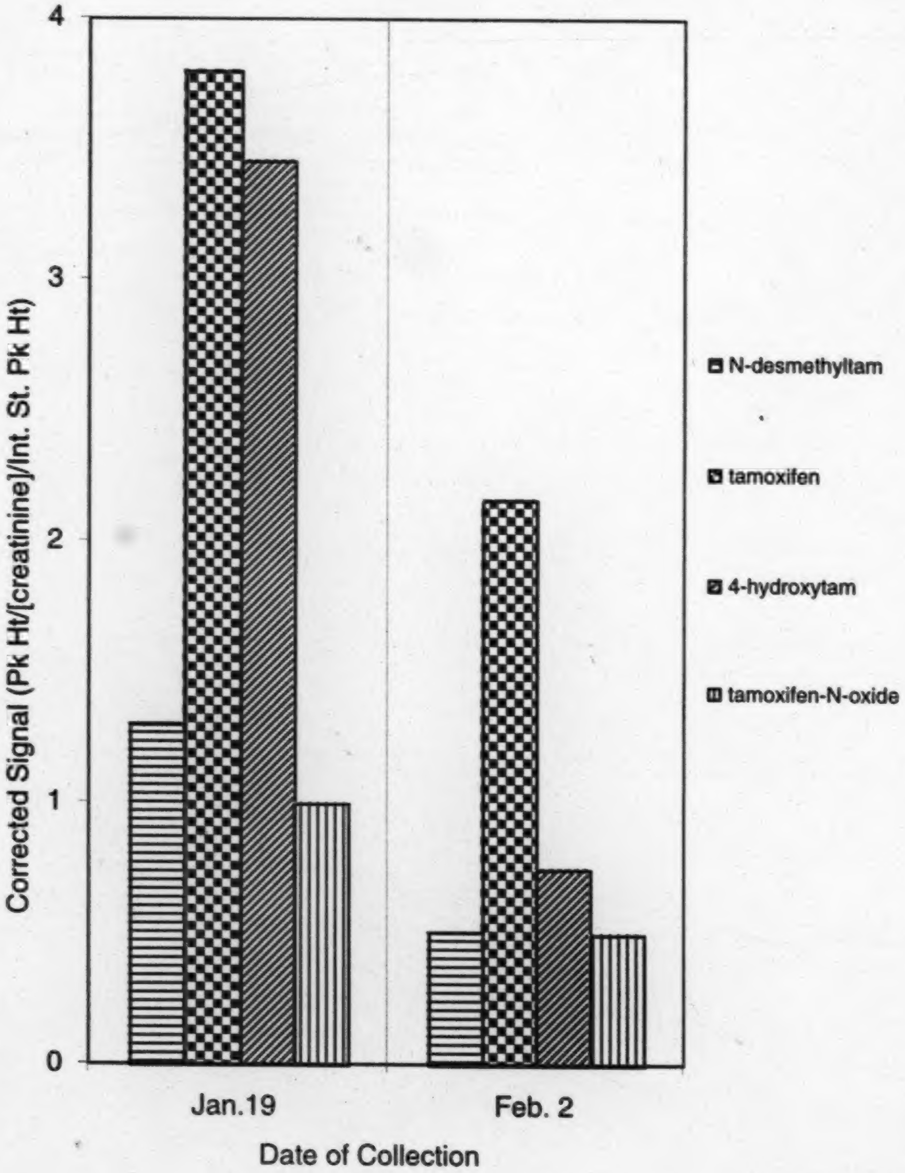
Figure 3.6. Urinary metabolic profile variation within the same day (second example).



3.3.3.2 Samples Over Longer Time

Does a patient's urinary metabolic profile alter over a two-week period? To answer this, three samples were collected from eight patients on one day and the same procedure was repeated two weeks later (same data in Section 3.3.3.1). Figure 3.7 shows how a urinary metabolic profile for one patient varies over two weeks. There is little variation, other than the increase in the tamoxifen to 4–hydroxytamoxifen ratio, in urinary metabolic profile for patients. Figure 3.7 shows the urinary metabolic profile for a stage II patient who had been taking tamoxifen for almost 16 months. Tamoxifen was found in greatest abundance, followed in order by 4–hydroxytamoxifen, N–desmethyltamoxifen, and tamoxifen–N–oxide. No 3,4–dihydroxytamoxifen was found. Again, the means and standard deviations for metabolites were calculated, with results being pooled and divided into groups. Each group consisted of the metabolite and the time of day the sample was collected (e.g. tamoxifen morning, tamoxifen afternoon, tamoxifen evening, N–desmethyltamoxifen morning, etc.). The means from each group on the first day were compared to the corresponding mean on the second day. (e.g. tamoxifen morning, Day1 and tamoxifen morning, Day 2; tamoxifen afternoon, Day 1 and tamoxifen afternoon, Day 2, etc.). A paired t-test was used to determine whether there were any significant differences between the means. With tamoxifen and four metabolites for three different times of day, 15 of these tests were performed. Out of these 15 tests, only one significant correlation was found: 3,4–Dihydroxytamoxifen, afternoon, Day 1 significantly correlates with 3,4–Dihydroxytamoxifen, afternoon, Day 2 ($p=0.024$). An additional paired t-test was also used to compare the differences of two means with standard deviations for each group on different days to give a p-value. Paired t-tests were done for different combinations of groups besides the corresponding same time of day (e.g. difference of tamoxifen morning, Day1 and tamoxifen afternoon, Day 1 compared to the difference of tamoxifen morning, Day 2 and tamoxifen afternoon, Day 2, etc.). Fifteen paired t-tests

Figure 3.7. Urinary metabolic profile variation over two weeks.



were performed, with no significant correlations found. Table 3.2 shows some of the statistics of these p-values. Because only one significant correlation was found from all of these tests, it is reasonable to conclude that a patient's urinary metabolic profile does not significantly alter over a two-week period.

3.3.4 Interpatient Metabolism Comparison

3.3.4.1 Patient Characteristics

Table 3.3 is a summary of patient characteristics. No patient was taking other medications known to modify tamoxifen metabolism (see Appendix II).

3.3.4.2 Patient Metabolic Profiles

Figures 3.8 and 3.9 present extracted ion electropherograms for urinary metabolites from patients with cancer stages I and IV respectively. In Figure 3.8 the strongest peak signal was the internal standard, amitriptyline (m/z 278), followed by 3,4–dihydroxytamoxifen (m/z 404), N-desmethyltamoxifen (m/z 358), 4–hydroxytamoxifen (m/z 388), tamoxifen (m/z 372), and tamoxifen–N–oxide (m/z 388). All peaks were well resolved, other than overlapping of 3,4–dihydroxytamoxifen and tamoxifen–N–oxide. Note the close, but different, migration times of tamoxifen and 4–hydroxytamoxifen. The 6 peaks migrated in a range from 7.5 to 11 minutes.

In Figure 3.9, except for 3,4–dihydroxytamoxifen and tamoxifen–N–oxide, peak signals are stronger than those in Figure 3.8. The strongest peak signal was N–desmethyltamoxifen (m/z 358), followed by tamoxifen (m/z 372), the internal standard, amitriptyline (m/z 278), 4–hydroxytamoxifen (m/z 388), 3,4–dihydroxytamoxifen (m/z 404). No tamoxifen–N–oxide (m/z 388) was detected. All peaks were well resolved. The peaks migrated in a range from 7.5 to 11 minutes.

Table 3.2. Statistics of p-values from urine tests. These tests were used to determine significant correlations between urinary metabolic profiles from the same patients over a two-week period. Metabolites that were analyzed were N–desmethyltamoxifen, tamoxifen, 4–hydroxytamoxifen, tamoxifen–N–oxide, and 3,4–dihydroxytamoxifen.

	Compare means	**Differences of means**
Frequency	15	15
Mean	0.41	0.59
Standard deviation	0.29	0.30
Median	0.29	0.54
Minimum	0.02	0.12
Maximum	0.93	0.96
Number of Significant tests ($p < 0.05$)	1	0

Table 3.3. Patient characteristics distributed by cancer stage.

Cancer Stage	Number of Patients		Age (years)	Weight (kg)	Height (cm)	Intake Duration (days)
I	3	Mean	57	65	156	426
		Minimum	46	57	154	227
		Maximum	65	73	158	586
II	26	Mean	57	79	161	615
		Minimum	37	55	151	97
		Maximum	86	117	174	1573
III	2	Mean	57	108	161	444
		Minimum	51	106	156	146
		Maximum	63	110	165	742
IV	16	Mean	57	72	161	971
		Minimum	38	51	149	35
		Maximum	82	115	175	3021
All	47	Mean	57	77	160	717
		Minimum	37	51	149	35
		Maximum	86	117	175	3021

Figure 3.8. Extracted ion electropherograms (from top to bottom: m/z 388, m/z 404, m/z 278, m/z 372, m/z 358) for stage I locally confined cancer patient.

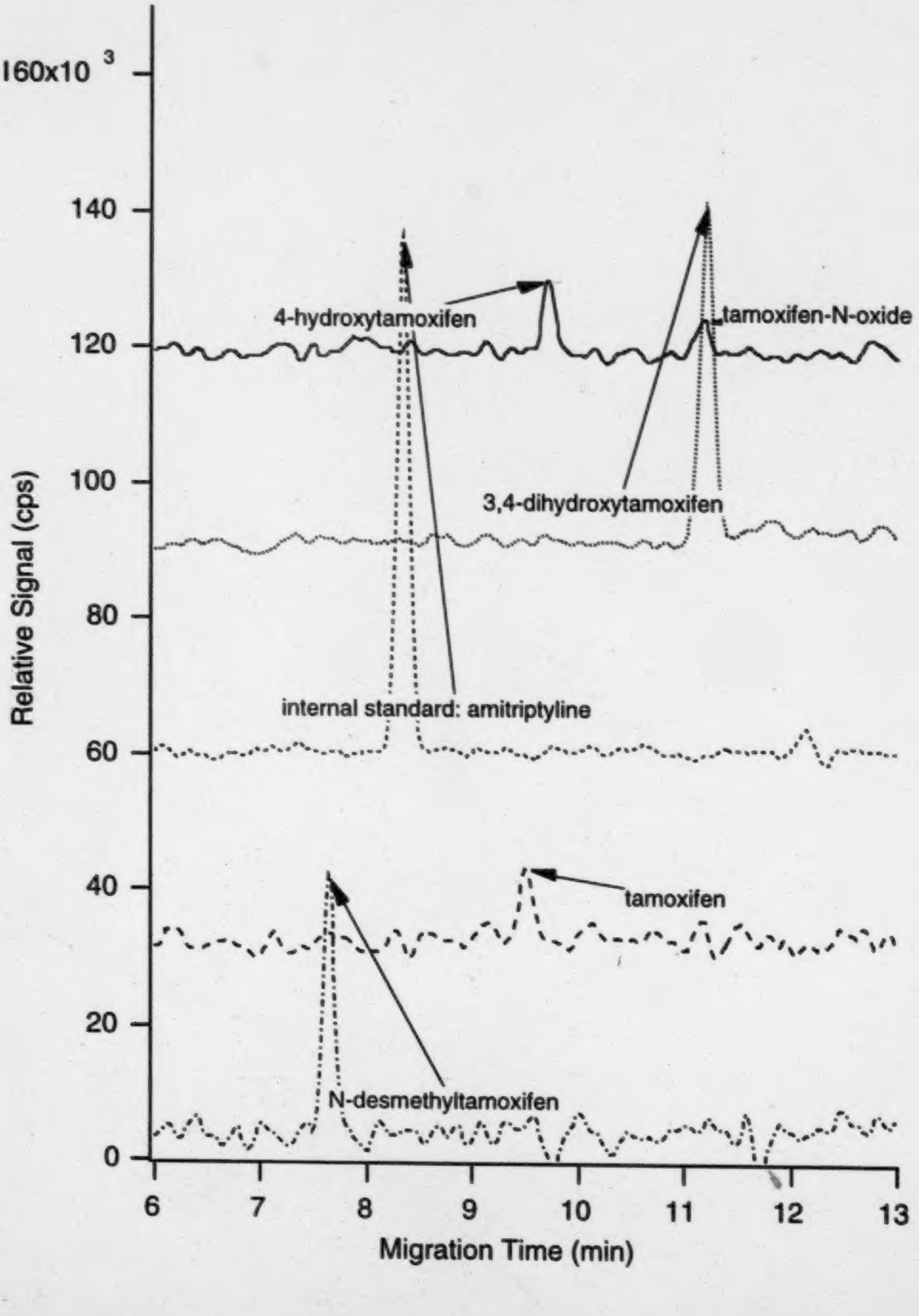
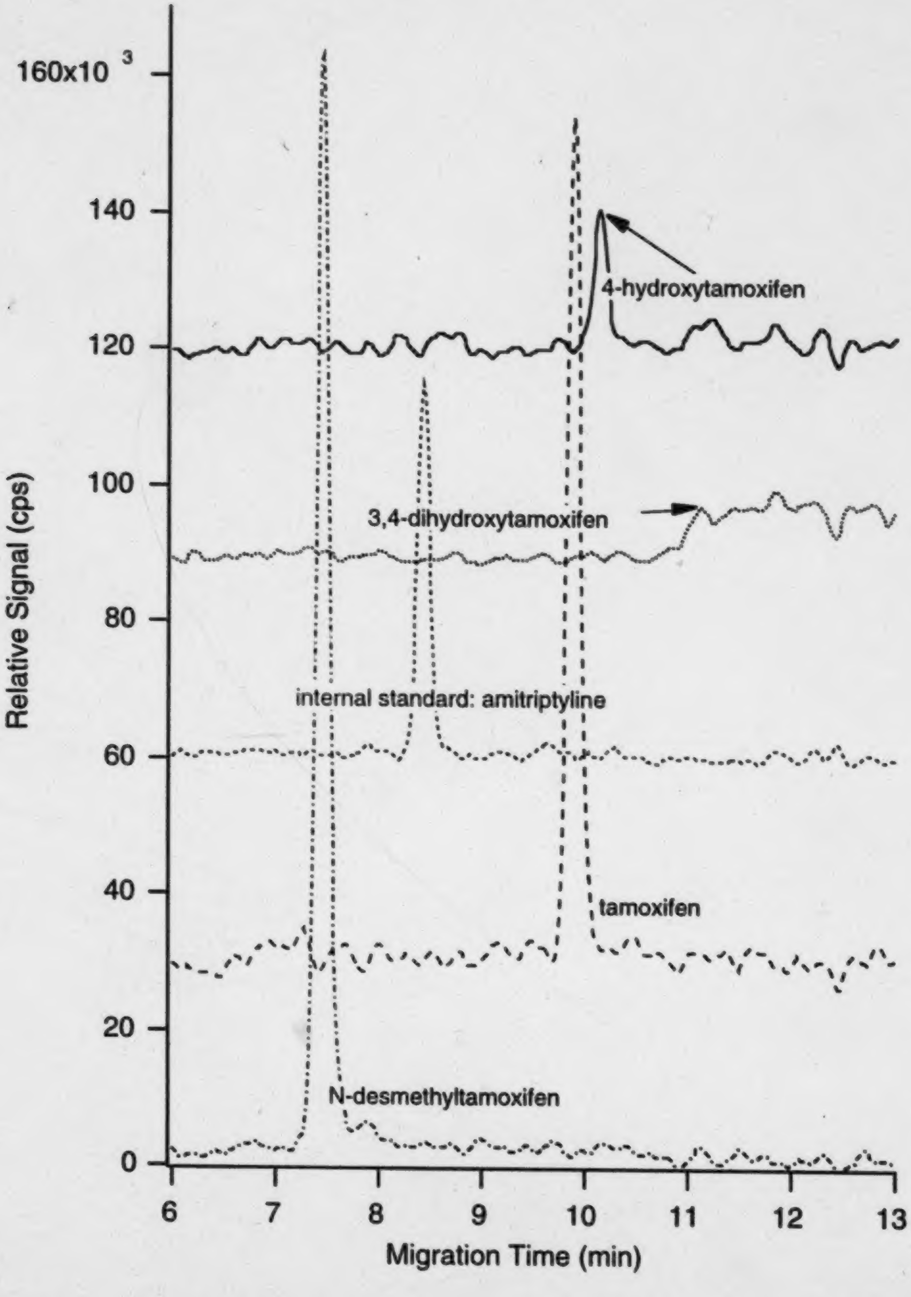


Figure 3.9. Extracted ion electropherograms (from top to bottom: m/z 388, m/z 404, m/z 278, m/z 372, m/z 358) for stage IV metastatic cancer patient.



These electropherograms are not necessarily reflective of other stage I or IV patient results, although it is indicative of the variation in metabolic profiles existing between patients. Figure 3.10 shows more clearly this metabolite production variation between patients by showing metabolite profiles from five patients in the form of a bar graph. These patients were chosen to demonstrate such wide variation.

In Figure 3.10, tamoxifen, N-desmethyltamoxifen, 4–hydroxytamoxifen, 3,4–dihydroxytamoxifen, and tamoxifen–N–oxide were analyzed and found in some of these five patients. Patient 5 had the largest signal when summing the metabolites and Patient 2 was the smallest. Only trace amounts of 4–hydroxytamoxifen were found in Patient 2. The sum of the metabolites analyzed in Patient 5 was 161 times greater than those in Patient 2. The RSDs of peak heights between these five samples were 78% for tamoxifen, 130% for N–desmesthyltamoxifen, 133% for 4–hydroxytamoxifen, and 112% for 3,4–dihydroxytamoxifen. No tamoxifen–N–oxide was detected.

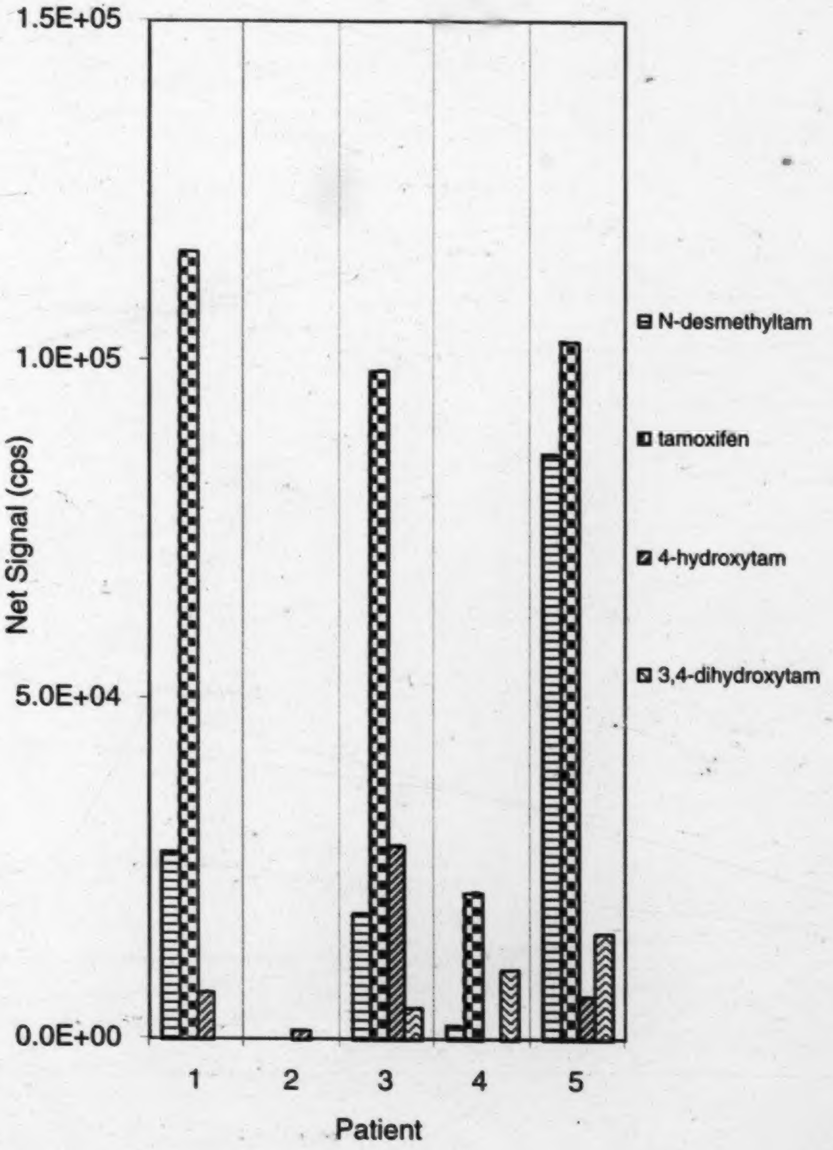
This large variation between patients stimulated questions about why variation existed and whether patient clinical characteristics were correlated to differences in metabolite production. To answer these questions, the experiment developed into a much larger scale.

For the blank sample from a healthy volunteer not taking tamoxifen, no peaks were found for any of the metabolites.

3.3.4.3 Relationships between Variables

Chi-square and Spearman correlation tests were used to analyze relationships among variables (see Appendix III). Chi-square tests were used to assess the association between metabolite levels and cancer stage (I, II, and III vs. IV) and the association between metabolite levels and disease progression for stage IV patients. Spearman rank correlation tests were used to determine the significance of the association between

Figure 3.10. Interpatient metabolic profile comparison of five patients.



metabolite levels and prognostic variables (age, weight, height, and duration of tamoxifen intake). In addition, this test was also used to determine the association among the metabolites themselves. Results with a p-value of less than 0.05 indicate significance. Two significant associations of tamoxifen and its metabolites in urine were found: N–desmethyltamoxifen associates with tamoxifen (R=0.48 p=0.0007) and N–desmethyltamoxifen associates with 4–hydroxytamoxifen (R=0.39, p=0.0070).

Interestingly, there was a significant association between cancer stage (I, II, and III vs. IV) and tamoxifen–N–oxide levels (p=0.039). A greater percentage of stage IV patients (those with known macroscopic metastatic tumors) had elevated tamoxifen–N–oxide levels compared to patients with cancer stages I, II, and III (those patients receiving 'adjuvant' tamoxifen therapy in the absence of macroscopic metastatic tumors). Another interesting result was a significant association between disease progression (progressors vs. non–progressors) and 3,4–dihydroxytamoxifen levels (p=0.041) for stage IV cancer patients. Of the 16 stage IV cancer patients, 6 were progressors. A greater percentage of progressors (those whose cancer has progressed despite continuing tamoxifen treatment) had elevated 3,4–dihydroxytamoxifen levels compared to non-progressors (those who are continuing to benefit from tamoxifen treatment).

No correlations were found between metabolite production and patient age, weight, height, and duration of tamoxifen intake.

3.4 Discussion and Conclusions

Although many explanations have been proposed for tamoxifen resistance in patients (15), the mechanisms for clinical resistance to tamoxifen are still not yet fully established (13-19,22). Among the leading explanations for acquired tamoxifen resistance is variability in tamoxifen metabolism among patients and within breast tumors. Osborne *et al.* have demonstrated a wide range of intertumoral tamoxifen levels and accumulation of the less antiestrogenic E isomer of 4–hydroxytamoxifen in some patients on tamoxifen therapy (16,17) and this has been proposed to be a mechanism of acquired tamoxifen resistance. Other studies have failed to demonstrate a relationship between serum levels of tamoxifen and major metabolites and *de novo* tamoxifen resistance (23,24). Because tamoxifen and its metabolites are primarily excreted by the biliary route only small quantities of these compounds can be observed in urine. In addition, the relationships between serum and urine metabolite profiles are not well studied. Nonetheless, several interesting correlations between patients' clinical characteristics and urinary tamoxifen metabolite profiles were observed.

Patients with stage IV breast cancer have macroscopic metastatic tumors. The stage IV patients in the study population had substantially higher tamoxifen–N–oxide metabolites than those patients with earlier-stage disease who had no evident macroscopic metastases. This observation raises the possibility that the breast cancer itself might influence tamoxifen metabolism, either directly by intratumoral metabolism, or indirectly by means of paraneoplastic effects on renal or hepatic function.

Of particular interest was the observation that stage IV cancer patients deriving prolonged benefit from tamoxifen (stage IV non-progressors) had lower 3,4–dihydroxytamoxifen levels than those stage IV patients whose cancer had progressed while on tamoxifen treatment (stage IV progressors). Although this suggests that tamoxifen urinary metabolism profiles may be predictive of tamoxifen efficacy, this observation should be considered preliminary due to the relatively undefined relationships between urine, serum, and intratumoural levels.

The use of nonaqueous CE/ESI/MS has been validated as a qualitative and quantitative technique for clinical analysis of tamoxifen and its metabolites in urine samples of breast cancer patients.

3.5 References

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3.6 Appendices

Appendix I. Corrected signal (Pk Ht/[Creatinine]/Int. Std. Pk Ht) data for 8 patients used to determine changes in urinary metabolic profiles within same day and over time.

Patient	Collection Date	Time of Day	N-desmethyl tamoxifen	tamoxifen	4-hyroxy tamoxifen	tamoxifen-N-oxide	3,4-dihydroxy tamoxifen
E148422	1/19/00	0800	0.23468806	2.86985937	1.04566762	0.52152902	0.68250133
		1600	6.6145749	21.3031039	4.97867746	3.72037787	4.48421053
		2400	6.84795576	19.6828072	1.55377128	2.20368512	2.1849376
	2/2/00	0800	2.37383292	9.68532638	0.75015289	0.71847352	0.47217352
		1600	0.00702195	0.01819095	0.02360307	0	0.00535647
		2400	0.01305607	0.05643016	0.01191979	0	0.00380022
E203481	1/12/00	0800	0.31195382	3.76964679	3.53570919	0	5.80656929
		1600	0.23370417	1.20842978	0.7534533	0.37359068	4.26943179
		2400	0.1918418	1.57570692	1.28257547	0.46350783	8.95448032
	1/26/00	0800	1.22630078	1.610335	2.19465431	0.56086957	0.78681397
		1600	0	0.30802896	0	0	0.21651946
		2400	0.69426921	1.60488672	2.00533096	0.35992892	0.41776988

E213876	1/19/00	0800	6.98531534	26.9986419	3.79500891	0.73351159	8.63093116
		1600	0	16.1713874	1.34459914	0.51305809	2.75285646
		2400	0	2.9238961	0.16359307	0.29350649	1.49320346
	2/1/00	0800	1.5216514	3.03104144	1.39795126	1.80024833	1.211237
		1600	8.4814436	0	0	3.08445354	0.77104033
		2400	1.53644598	2.168967	2.56103084	0.64082381	0.76493422
E225954	12/1/99	0800	2.57887764	11.5626005	3.5397937	0	0.67012397
		1600	1.29111869	18.5992856	7.59961033	0	0.78210748
		2400	1.93591202	4.29128218	5.86124805	2.88522311	0.56410905
	12/15/99	0800	0	1.18935103	0.29645034	0.1180342	0.09881858
		1600	0	0	0	0	0
		2400	0	4.78056841	0	3.1075347	1.31011236
E228354	1/12/00	0800	0	0	0	2.84343252	1.45752576
		1600	3.33014051	11.7070553	0	0.57418658	0.38910216
		2400	0.82026359	19.18865	1.03181307	0	0.46422887
	1/25/00	0800	6.23941625	6.3899553	3.24533263	1.29042861	1.31567184
		1600	5.60190314	12.7704871	2.06746201	1.35492118	1.61823605
		2400	0.98820782	5.57174548	2.73613543	1.03385873	1.10297723
E229268	12/3/99	0800	0.54452872	3.20299157	0.9858378	0	0.47843844
		1600	11.4420939	28.8182144	3.42822995	9.56949922	1.55396199
		2400	0.504675	3.21065675	1.16880703	0	0.24048665
	12/16/99	0800	0.43861696	4.38806749	0.15107852	0.08596959	0.05630229
		1600	61.7133646	24.1617819	0	11.2315358	0
		2400	26.8298511	45.6336795	2.70499187	1.06380583	0.721719

E241574	1/19/00	0800	13.3354023	26.1703103	1.86028859	0.60952757	3.80185807
		1600	0	0.26816324	0	0.67453202	1.47895001
		2400	1.4383851	8.78134414	8.94470413	0.432672	4.66282331
	2/2/00	0800	2.84096163	6.99714902	1.57805517	0	0.51641239
		1600	16.8264928	15.6914717	6.33883135	1.52870477	1.91215957
		2400	6.88900118	20.104093	3.3365336	1.37695806	1.56392117
E245526	1/19/00	0800	1.26709193	3.13610383	1.96621088	0.19613478	2.08286229
		1600	1.29813021	3.80260674	3.45672696	0.99738108	5.85388879
		2400	0	0.33710099	0.20999477	0.3700157	1.42919937
	2/2/00	0800	0	0.21022956	1.62714458	0.65420862	0.3255739
		1600	0.50665726	2.16092055	0.74631867	0.50268936	0.83810951
		2400	1.30632785	1.22899851	2.4941704	1.26058794	1.00149477

Appendix II. Patient clinical characteristics for urine analyses.

Patient	Initial Diagnosis	Tamoxifen Prescription	Cancer Stage	Date of Advanced/ Metastatic disease	Progression Date	Age (years)	Height (cm)	Weight (kg)
C119926	?	7/9/98	4	?	none	44	160	62.2
E085625	3/19/97	11/27/97	2	n/a	n/a	46	166	86
E107381	4/30/97	5/12/98	4	at diagnosis	none	52	175	86.5
E122887	?	9/14/98	4	?	none	69	?	73
E141395	?	2/11/97	2	n/a	n/a	62	162	90
E148422	12/11/98	3/5/99	2	n/a	n/a	62	175	105
E158163	?	1/28/97	4	?	none	75	165	74
E162798	?	3/16/99	4	?	7/29/99	50	162	60.1
E164423	?	6/11/99	2	n/a	n/a	50	151	65
E184840	?	11/13/91	4	?	none	82	158	55
E188374	?	4/6/99	4	?	5/10/99	61	156	61
E195134	?	1/11/91	4	?	none	73	158	62.5
E195513	?	12/28/92	4	?	none	86	160	65
E203481	9/28/98	1/18/99	1	n/a	n/a	46	158	56
E210587	?	12/16/94	2	n/a	n/a	58	168	89
E213876	7/22/99	1/16/99	2	n/a	n/a	75	164	78
E217556	?	12/7/95	2	n/a	n/a	56	160	73
E217558	?	9/26/95	2	n/a	n/a	56	166	79

E222736	?	9/25/96	2	n/a	n/a	54	153	89
E225630	?	9/27/96	2	n/a	n/a	61	155	70
E225954	5/28/96	7/2/96	2	n/a	n/a	44	148	56
E228354	4/16/97	12/8/97	2	n/a	n/a	46	155	65
E229268	8/27/97	8/17/99	4	8/5/99	none	73	?	74
E229568	?	6/29/98	4	?	1/29/99	52	149	51.2
E230851	?	6/10/97	4	?	9/14/99	51	165	115
E232497	?	2/20/97	2	n/a	n/a	51	162	65
E232587	?	8/26/96	2	n/a	n/a	58	152	60
E234321	9/27/96	10/23/96	2	n/a	n/a	59	?	67
E234712	?	4/8/97	3	n/a	n/a	52	165	110
E235807	?	4/12/97	2	n/a	n/a	63	155	100
E236944	?	6/5/97	2	n/a	n/a	60	170	93
E236972	?	12/11/97	4	?	4/27/99	54	159	82
E237435	?	5/1/98	2	n/a	n/a	54	157	86.4
E238637	?	1/25/98	4	?	none	46	166	79
E238659	?	9/10/97	1	n/a	n/a	66	157	64
E239174	?	12/8/97	2	n/a	n/a	76	?	70
E239840	10/7/97	1/22/98	1	n/a	n/a	60	154	73
E240518	?	5/12/99	2	n/a	n/a	38	?	?
E240812	?	7/2/98	2	n/a	n/a	45	161	55
E241574	1/17/97	1/11/99	2	n/a	n/a	45	167	68
E242016	?	8/11/98	2	n/a	n/a	38	161	88
E243107	?	10/19/98	2	n/a	n/a	51	161	58
E243360	?	10/13/98	2	n/a	n/a	62	161	86

E243834	?	12/8/98	3	n/a	n/a	63	156	106
E244147	?	11/3/98	2	n/a	n/a	55	160	67
E245024	?	9/30/98	2	n/a	n/a	75	165	93
E245526	8/14/98	9/23/98	2	n/a	n/a	75	160	56
E246945	?	11/27/98	4	?	none	61	159	78
E248700	?	5/26/99	2*	n/a	n/a	87	151	80
E251319	?	1/13/99	2	n/a	n/a	54	174	117
L023120	?	4/25/98	2	n/a	n/a	62	?	?
R001839	?	6/30/98	4	?	9/13/99	38	163	73

Appendix III. Corrected signal (Pk Ht/[Creatinine]/Int. Std. Pk Ht) data used to determine associations between patient clinical characteristics (see Appendix II) and urine metabolic profiles.

Patient	**N-desmethyl tamoxifen**	**tamoxifen**	**4-hydroxy tamoxifen**	**tamoxifen-N-oxide**	**3,4-dihydroxy tamoxifen**
C119926	0	0	4.67202061	0	0
E085625	109.041729	84.268014	14.0704229	0	10.0797994
E107381	10.3566667	12.504375	8.31729167	0	0
E122887	41.7193454	25.9822459	2.01371613	0	4.16012739
E141395	2.73704755	1.98343558	2.2875	0	2.96953988
E158163	2.07519347	0	0	21.1902194	0
E162798	12.548461	65.995535	19.1085387	0	3.0394312
E164423	32.3428898	28.4402306	12.1396406	0	65.6870964
E184840	0	21.576661	2.78138413	0	0
E188374	14.8300729	31.0429179	1.31418112	0	1.00638607
E195134	154.086319	376.576651	27.3823107	0	14.6047617
E195513	43.1644089	13.9389864	0	0	5.02309702
E203481	30.798944	50.5346447	0	0	136.203083
E210587	0	0	2.17527611	2.08007803	5.51077881
E217556	0	0	0	0	60.6958219
E217558	17.7669762	32.255747	38.8994821	0	13.9152619

E222736	11.087254	40.337647	0	0	13.4827359
E225630	0	27.8809529	0	0	30.6877603
E229568	182.16125	0	0	0	127.71875
E230851	64.0297949	88.1643549	16.5063412	0	5.74997392
E232497	72.2584342	306.617879	18.7889328	0	0
E232587	4.8610314	19.5329963	0	0	0
E234321	89.7872917	107.088746	6.57979238	0	16.2306397
E234712	0	0	0	0	31.4328154
E235807	6.6168413	8.99843108	0	0	12.333732
E236944	0	87.2109916	0	0	283.913073
E236972	14.0168922	128.651702	0	39.2049585	40.8415518
E237435	8.9609	18.60195	13.119575	16.83295	0
E238637	0	2.18970457	1.9179438	0	0
E238659	0	61.945421	0	0	6.46623705
E239174	1.87695983	18.9013607	0	0	9.04535824
E239840	0	0	0	0	0
E240518	47.0622218	30.6055648	0	0	10.8630296
E240812	0	3.3354223	4.02751126	2.87105293	2.54658971
E242016	0	0	13.9542426	0	11.9411956
E243107	14.245509	83.8941766	11.6931886	0	0
E243107	0	11.2696632	0	0	9.30842323
E243360	24.6335343	56.1630588	9.62225163	0	0.72691667
E243834	23.5472039	63.8092105	0	0	10.7994079
E244147	10.7452935	41.4498751	4.82533839	5.44542712	7.89084748
E245024	0	0	0	0	324.332102

E246945	0	637.98129	0	0	0
E248700	169.685648	66.3103715	7.34152613	0	10.9502302
E251319	11.8183463	53.2895883	0	0	46.0566252
L023120	10.2575207	52.5964917	39.7431008	8.85827003	55.9700276
R001839	0	938.527891	0	0	306.667969
R001839	0	0	0	0	129.488892

Chapter 4

Analysis of Tamoxifen and Its Metabolites in Serum from Breast Cancer Patients

4.1 Introduction

Many analyses of tamoxifen biotransformation in human serum or plasma have been performed. Some of the more concentrated tamoxifen metabolites found in serum include N–didesmethyltamoxifen, N–desmethyltamoxifen, tamoxifen, N–desmethyl–4–hydroxytamoxifen, and 4–hydroxytamoxifen. The techniques for these analyses include high performance liquid chromatography (HPLC) with ultraviolet (UV) absorbance or fluorescence detection (1-10) and HPLC with electrospray mass spectrometry (ESI/MS) detection (11). Capillary electrophoresis (CE) with UV detection has also been used to analyze tamoxifen and its metabolites in serum samples (12). According to literature searches, nonaqueous CE/ESI/MS has never been used to analyze tamoxifen and its metabolites in serum.

Nonaqueous CE/ESI/MS was previously used to analyze tamoxifen and its metabolites in urine samples from breast cancer patients undergoing tamoxifen treatment (see Chapter 3). The following were observed: Substantial variation among patient profiles, statistically significant differences in the amount of urinary tamoxifen N-oxide found in patients with breast cancer stages I, II, or III compared to stage IV patients, and statistically significant differences in the amount of 3,4–dihydroxytamoxifen found in metastatic patients continuing to benefit from tamoxifen treatment compared to metastatic patients whose cancer had progressed. Explanations for these observations were proposed. In this chapter, serum samples are analyzed for the purpose of continuing to (i) investigate how tamoxifen is metabolized in humans and confirm the hypothesis of differential metabolism in individual patients, and to (ii) explore relationships between patient serum tamoxifen metabolic profiles and clinical characteristics.

4.2 Experimental

4.2.1 Chemicals

(Z)-Tamoxifen was purchased from Sigma (St. Louis, MO, U.S.A.). (Z)–4–hydroxytamoxifen was purchased from Research Biochemical International (Natik, MA, U.S.A.). (Z)-N-desmethyltamoxifen was obtained from Sciex (Concord, ON). (Z)–4–hydroxytamoxifen–N–desmethyltamoxifen was obtained from Ken Roberts at Iowa State University. N–Didesmethyltamoxifen was identified by in-source collision induced dissociation (see Figure 2.3). HPLC–grade methanol and acetonitrile were purchased from Caledon (Georgetown, ON) and EM Science (Gibbstown, NJ, U.S.A.), respectively. Ammonium acetate was purchased from ACP Chemicals (Montreal, PQ). Tamoxifen and metabolite stock solutions (1000 μg/mL each) were prepared by dissolving directly into methanol. Standard solutions (0.001-1 μg/mL) were then prepared by serial dilution with methanol. The CE running buffer was 5 mM ammonium acetate in methanol-acetonitrile (70:30).

4.2.2 Serum Samples

Serum samples were obtained from eighty patients taking 20 mg of tamoxifen (Nolvadex, Astra-Zeneca) daily by mouth. All patients had been diagnosed with invasive ductal carcinoma of the breast (stages I, II, III or IV) and were being followed at the Cross Cancer Institute (Edmonton, AB). All patients had estrogen receptor positive disease determined by immunohistochemistry using antibody H226. Samples were stored at –20°C until analysis. All patients gave informed consent and the Cross Cancer Institute Research Ethics Committee approved the research protocol. All assays conducted were blind with regards to clinical characteristics of the patients. A blank serum sample from an advanced metastatic stage breast cancer patient not undergoing tamoxifen treatment was analyzed.

4.2.3 Extraction of Metabolites

Similar to the procedures that Poon *et al.* (11) reported, tamoxifen metabolites were extracted from 500 µL of serum with hexane containing 2% butanol (3 x 2.5 mL). Imipramine (Sigma, St. Louis, MO, U.S.A.), an antidepressant drug with a similar structure to tamoxifen metabolites, was added to serum as an internal standard before extraction. The organic layers from the extract were combined and dried using vacuum centrifugation. The dry residues were kept at –20°C. Samples were redissolved in 100 µL methanol just prior to CE injection.

4.2.4 Capillary Electrophoresis

An in-house constructed CE system was used for separations. Untreated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, U.S.A.) with 60 cm x 50 µm i.d. x 150 µm o.d. dimensions were used for separations. Fresh running buffers were used to flush the capillary before and between runs. Electrokinetic injections at 10 kV for 5 s were used to introduce samples into the capillary. Separations were performed at 20 kV. The operating electrolyte was changed every run to avoid contamination and solvent evaporation. All runs were done at ambient temperature.

4.2.5 Electrospray Ionization Mass Spectrometry

A PE/Sciex API 100 single quadrupole mass spectrometer equipped with an ion spray source was used to analyze eluents from the online CE. The CE column was inserted 0.5–1.0 mm inside the tip of the ESI electrode, and the electrode was 0.5–1.0 mm outside the nebulizer tube. A Harvard Apparatus (South Natick, MA, U.S.A.) low-pressure syringe infusion pump was used to pump methanol, the coaxial sheath liquid. The flow rate was 2.0 μL/min through a capillary of 60 cm x 50 μm i.d. x 50 μm o.d. dimensions. Positive ion mode was used for all measurements. Mass spectrometer conditions were optimized frequently with direct infusion of a polypropylene glycol polymer standard (PE Applied Biosystems, Foster City, CA, U.S.A.) and then with tamoxifen standard. Ion spray voltages ranged from 4700 to 5200 V, orifice voltages from 35 to 40 V, ring voltages from 140 to 180 V, nebulizer gas flow rates from 0.17 L/min (NEB 1) to 1.04 L/min (NEB 8), curtain gas flow rates from 0.95 L/min (CUR 7) to 1.25 L/min (CUR 10), and CEM voltages from 2400 to 3000 V. A working pressure of 1.1 x 10^{-5} Torr was maintained in the analyzer chamber. During sample injections, the ionspray voltage was set to 0 and the nebulizer gas flow rate was set to 0.03 L/min (NEB 0). For data acquisition the mass to charge range was 277-405 amu, step size was 1.0 amu, scan speed was 0.39 s, dwell time was 1.5 ms, and pause time was 3.0 ms. Electropherograms were acquired by LC Tune software (PE Sciex, Concord, ON), and then processed by Multiview 1.2 software from Sciex.

4.3 Results

4.3.1 Serum versus Plasma Analysis

Blood plasma consists of serum, protein, and low molecular weight substances in aqueous solution. If whole blood is prevented from clotting either by chilling it or by adding anticoagulants, such as sodium citrate, it can be centrifuged. The clear fluid that then occupies roughly the upper half of the centrifuge tube is called plasma. Blood serum can be obtained by allowing whole blood to clot, and after coagulation, agitating the plasma to precipitate the clotting factors as a large clot. The leftover watery fluid is serum (13).

To determine whether plasma or serum samples should be used for large-scale analysis of tamoxifen and its metabolites in blood, a plasma and serum sample were each obtained from two patients and compared. Figure 4.1 shows metabolic profiles from these analyses and these numbers are summarized in Table 4.1. Only four metabolites were analyzed. 4-Hydroxytamoxifen (m/z 388) was not detected in either patient. The net signal for the serum analyses were the same or greater than the net signal for the plasma analyses in both patients for N–didesmethyltamoxifen, N–desmethyltamoxifen, and tamoxifen. For N–didesmethyl–4–hydroxytamoxifen, the net signal was higher for plasma. Based on these results, it was decided that serum, not plasma, would be analyzed for the analysis of tamoxifen and its metabolites in blood.

4.3.2 Serum Extraction and Analysis Repeatability

Three replicates of the same serum sample were extracted and analyzed on the same day. The profiles of metabolites were repeatable as shown in Figure 4.2.

Figure 4.1. Comparison between plasma and serum metabolic profiles of two patients.

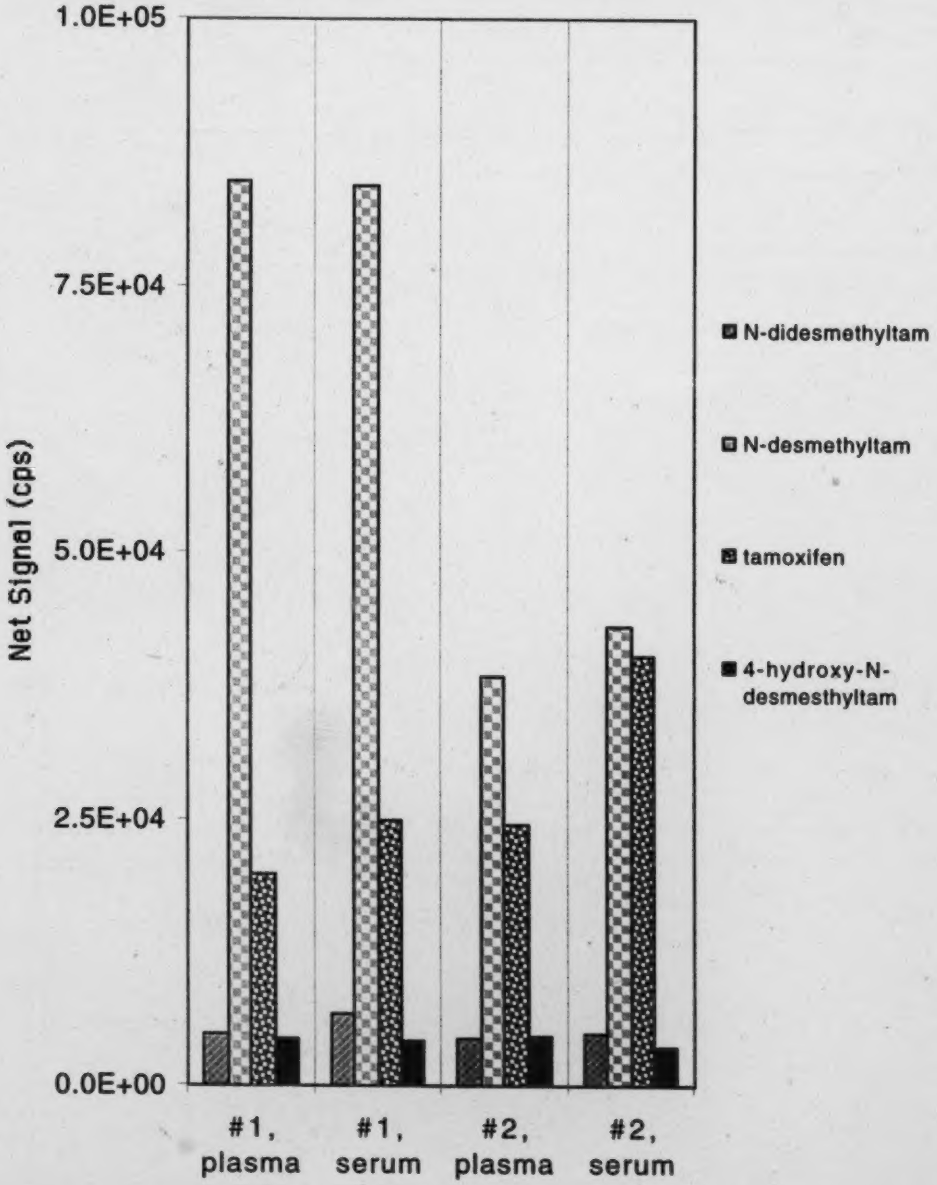
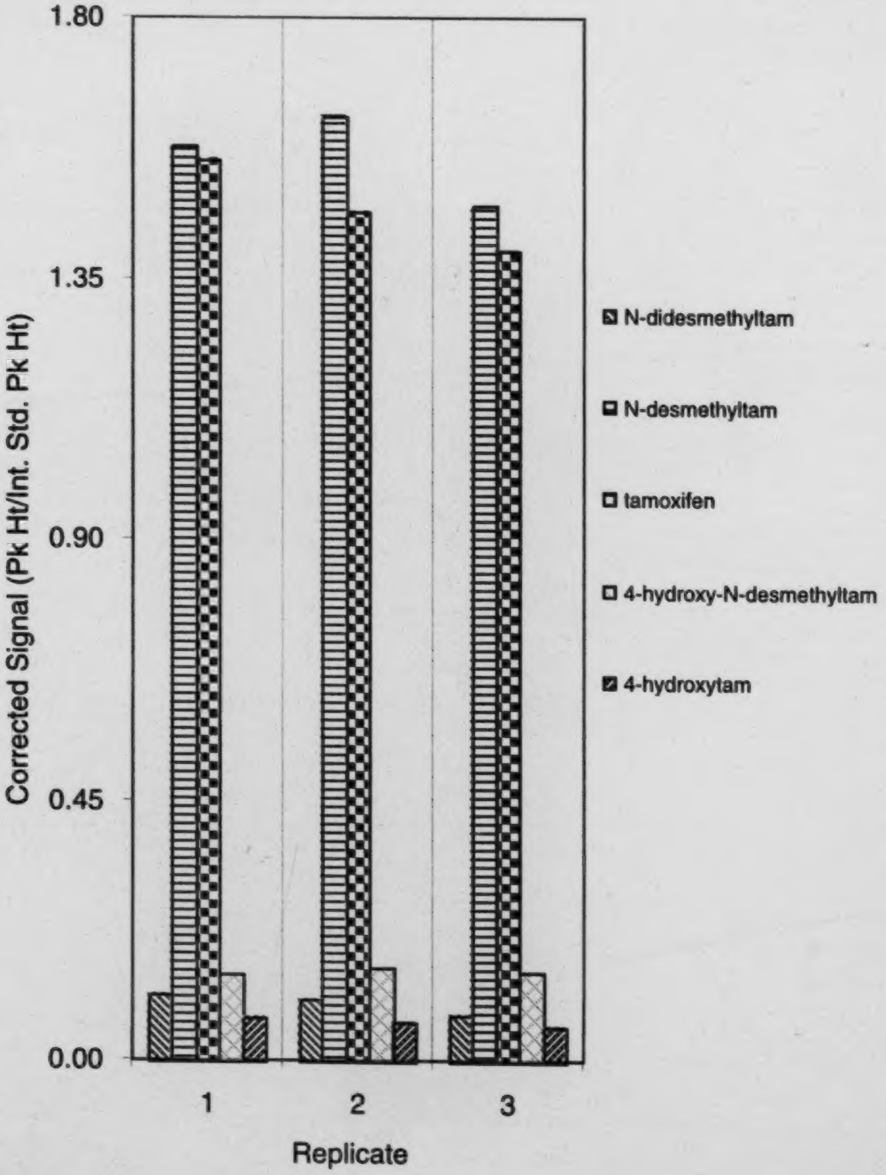


Table 4.1. Comparison of two patients net signal (in cps) from serum and plasma samples.

Patient	**Analysis**	**N–didesmethyl-tamoxifen**	**N–desmethyl-tamoxifen**	**tamoxifen**	**N–desmethyl–4–hydroxy–tamoxifen**
1	serum	6754	84447	24921	4299
	plasma	4914	84773	19812	4525
	s/p ratio	1.37	1.00	1.26	0.95
2	serum	4894	43030	40323	3617
	plasma	4508	38496	24563	4731
	s/p ratio	1.09	1.12	1.64	0.76

Figure 4.2. Repeatability of the entire analytical procedure: Three replicates from the same serum sample analyzed on the same day.



Relative standard deviation (RSD) values for the corrected signal (net peak height / imipramine internal standard peak height) are 18.7% for N–didesmethyltamoxifen, 4.9% for N–desmethyltamoxifen, 5.3% for tamoxifen, 4.0% for 4–hydroxy–N–desmethyltamoxifen, and 11.5% for 4–hydroxytamoxifen. The RSD values demonstrate satisfactory variations in the process from sample extraction to instrument analysis.

4.3.3 Changes in Patient Metabolic Profiles over Time

Similar to the urine analysis, the question was asked, "Do the metabolites in serum vary from day to day?" To answer this, 34 different patients gave blood two weeks apart (see Appendix IV). Tamoxifen and four metabolites in serum were analyzed (N–didesmethyltamoxifen, N–desmethyltamoxifen, tamoxifen, N–desmethyl–4–hydroxytamoxifen, and 4–hydroxytamoxifen). The statistical tests to determine any significant differences were done in two ways, similar to how the urine samples were analyzed (section 3.3.3.2). The means for each metabolite were compared on different days using the paired t-test. No significant correlations were found. Second, using the paired t–test, the differences between the two days for each metabolite in each patient were summed. In theory, a sum of zero would indicate no significant differences. Using p–values, no significant differences were found. Table 4.2 lists some of the statistics of the p-values from these 34 paired t-tests for each metabolite. These results are an indication that a patient's metabolic profile in serum does not significantly change over a two-week period.

Table 4.2. Statistics of p-values from paired t-tests of serum samples. These tests were used to determine significant correlations between serum metabolic profiles from the same patients over a two-week period. Metabolites that were analyzed were N–didesmethyltamoxifen, N–desmethyltamoxifen, tamoxifen, N–desmethyl–4–hydroxytamoxifen, and 4–hydroxytamoxifen.

	Compare means directly	**Differences of means**
Number of tests (each test compared 34 patients)	5	5
Mean	0.71	0.58
Standard deviation	0.17	0.24
Median	0.73	0.56
Minimum	0.52	0.30
Maximum	0.96	0.94
Number of Significant tests ($p < 0.05$)	0	0

4.3.4 Interpatient Metabolism Comparison

4.3.4.1 Patient Characteristics

Table 4.3 is a summary of patient characteristics. No patient was taking other medications known to modify tamoxifen metabolism (see Appendix V).

4.3.4.2 Patient Metabolic Profiles

Figure 4.3 presents a typical example of extracted ion monitoring electropherograms for metabolites in serum from a patient with stage II (surgically resected) breast cancer. The components are reasonably well resolved, and the separation is complete within 7.5 minutes. No metabolite peaks were detected in the blank serum sample from a metastatic breast cancer patient not undergoing tamoxifen treatment.

4.3.4.3 Relationships between Variables

Spearman rank correlation and multivariate regression analysis were performed with p–values of less than 0.05 indicating significance. Univariate and multivariate tests were used to assess associations between metabolite levels and patient characteristics (see Appendix VI). Significant associations on multivariate analysis were found for tamoxifen and N–desmethyltamoxifen levels in cancer stage and age. The first row in Table 4.4 indicates the distribution (subsequent rows contain the results for these two metabolites) of the division between early (stages I and II) and advanced (stages III and IV) breast cancer.

Table 4.3. Patient characteristics distributed by cancer stage.

Cancer Stage	Number of Patients		Age (years)	Weight (kg)	Height (cm)	Intake Duration (days)
I	11	Mean	63	77	159	524
		Minimum	46	48	154	149
		Maximum	82	120	165	1034
II	47	Mean	55	74	162	606
		Minimum	39	53	147	43
		Maximum	76	125	175	1791
III	5	Mean	55	88	158	966
		Minimum	47	67	151	649
		Maximum	65	101	164	1400
IV	17	Mean	59	78	161	450
		Minimum	41	48	150	119
		Maximum	93	115	173	914
All	80	Mean	57	76	161	584
		Minimum	39	48	147	43
		Maximum	93	125	175	1791

Figure 4.3. Extracted ion electropherograms (from top to bottom: m/z 388, m/z 344, m/z 374, m/z 372, m/z 358) of tamoxifen metabolites in serum from a patient with stage II (surgically resected) breast cancer.

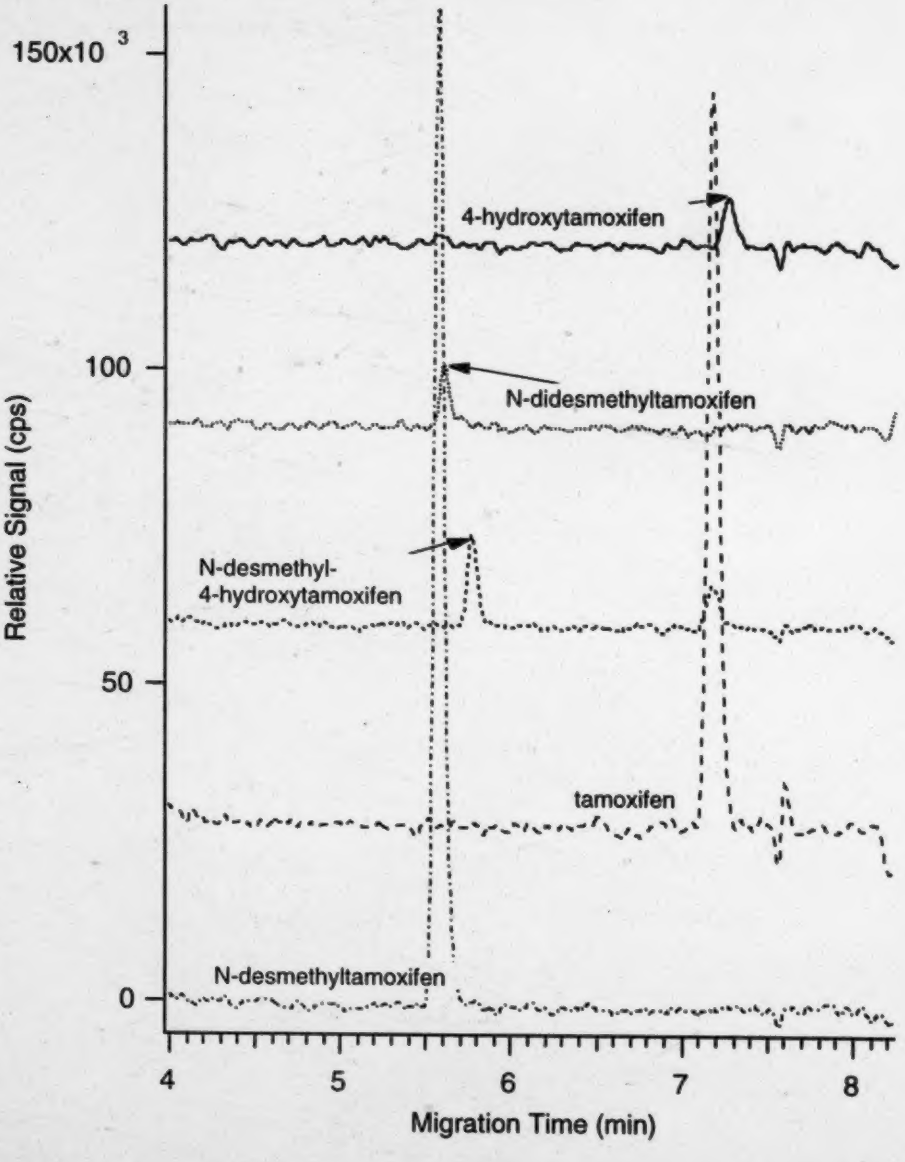


Table 4.4. Comparison of grouped cancer stages to tamoxifen and N-desmethyltamoxifen metabolite production.

	Stages I and II	**Stages III and IV**
Frequency	58	22
Mean tamoxifen corrected signal (+/- standard deviation)	2.04 +/- 1.03	1.48 +/- 0.64
Median tamoxifen corrected signal	1.85	1.43
Mean N-desmethyltamoxifen corrected signal (+/- standard deviation)	3.52 +/- 2.31	2.45 +/- 1.64
Median N-desmethyltamoxifen corrected signal	2.81	2.00

A multivariate regression analysis test of metabolite concentration with cancer stage (I and II vs. III and IV) and age was performed. A highly significant negative correlation for cancer stage and tamoxifen levels (p=0.0097) and N–desmethyltamoxifen levels (p=0.016) was observed. Applying the same test to the logarithms of these corrected signals gave similar results for tamoxifen (p=0.011) and N–desmethyltamoxifen (p=0.024). These logarithmic transformations were performed as an added measure to further validate these correlations. The reason for this is that irregularities, if any, in the distribution of signal (for example, just a few very high or low signals, compared to the other values, could change results) would not distort the results.

Multivariate analysis also identified a significant positive correlation between patient age and tamoxifen levels (p=0.018) and also N–desmethyltamoxifen levels (p=0.0003). Applying the same test to the logarithms of these corrected signals gave an insignificant result for tamoxifen (p=0.073), but the result for N–desmethyltamoxifen (p=0.016) remained significant. For the other metabolites, no significant correlations were found.

A Spearman rank correlation showed that concentrations of most serum tamoxifen metabolites were significantly correlated to each other as indicated in Table 4.5.

Cox regression analysis was used to explore the association between metabolite levels and the duration of clinical benefit experienced by those patients with stage IV disease. The time to disease progression from the initiation of tamoxifen treatment was determined for each of the 17 stage IV cancer patients, however, insufficient numbers of patients had experienced disease progression for meaningful analysis. Follow-up on all of

Table 4.5. Spearman rank correlation analysis of associations between tamoxifen and metabolites. Presented are the correlation coefficient and p values (in parentheses).

Metabolite	**N-didesmethyl tamoxifen**	**N-desmethyl tamoxifen**	**Tamoxifen**	**N-desmethyl-4-hydroxy tamoxifen**	**4-hydroxy tamoxifen**
N-didesmethyl tamoxifen	-				
N-desmethyl tamoxifen	0.78 (0.0001)	-			
tamoxifen	0.66 (0.0001)	0.79 (0.0001)	-		
N-desmethyl-4-hydroxy tamoxifen	0.81 (0.0001)	0.71 (0.0001)	0.74 (0.0001)	-	
4-hydroxy tamoxifen	Not significant	Not significant	0.46 (0.0001)	0.38 (0.0001)	-

these patients will continue and this data set will be reanalyzed with an additional year follow-up, and again in five years. This is expected to yield sufficient numbers of events (progression of advanced disease, or recurrence of early stage disease) to explore relationships between tamoxifen metabolite profiles and clinical outcomes.

Using univariate and multivariate regression analysis, no correlations were found between metabolite production and duration of tamoxifen treatment, patient weight, or height.

4.3.5 Serum and Urine Comparison

There is much variation between urinary metabolic profiles of different patients (see Section 3.3.4.2). The variation between patients in serum varies much less. What though of the variation between urine and serum from the same patient? To determine this, urine and serum samples from eight patients were collected at essentially the same time (one or two days apart). N–Desmethyltamoxifen, tamoxifen, and 4–hydroxytamoxifen were analyzed in both samples and compared. The difficulty with such a comparison is that the corrected signals for urine and serum are on different scales because an extra correction has to be made for urine samples using the creatinine standard (see Section 3.3.1). Nevertheless, comparisons can be made by testing for the significance of the Pearson correlation between urine and serum variables. It is a test of association rather than of comparison so the difference in scales is not a problem.

Because patients gave three samples of urine on the same day (same data used here as for Section 3.3.3.1), all three of those urine samples were each used in a comparison with the one serum sample that was collected at essentially the same time (same data as Section 4.3.3). Comparisons were done for one collection date and then patients repeated the same procedure two weeks later. For the first collection date, only three correlations (out of nine) were found to be statistically significant:

Urine, N–desmethyltamoxifen, morning/ Serum, N–desmethyltamoxifen
$R=0.82$
$p=0.013$

Urine, tamoxifen, morning/ Serum, tamoxifen
$R=0.73$
$p=0.042$

Urine, 4–hydroxytamoxifen, evening/ Serum, 4–hydroxytamoxifen
$R=0.86$
$p=0.0066$

For the second collection date, two weeks after the first, three urine samples and one serum sample were collected from the same eight patients. Statistical results for this second collection date showed no significant correlations. In fact the three correlations that showed significance on the first collection date had high p–values ($p=0.98$, 0.97, 0.71, respectively) in contrast to the second collection date. From these results, it is likely that no correlation exists between a patient's urine and serum metabolic profiles. In other words, the concentration of metabolites found in urine has no significant association to the concentration of metabolites found in serum for the same patient at any given time.

It was concluded that it is likely that both urine (section 3.3.3.2) and serum (section 4.3.3) metabolic profiles do not significantly change within a two-week period. Considering these conclusions, one might expect that the correlation between urine and serum metabolite concentrations should show a significant association. There are some possibilities why no significance was found: First, there is enough noise in the data to make correlations suspect. Second, there were only 8 patients that had their urine samples analyzed to test for metabolic profile changes compared to 34 for serum. The difference with these numbers may have affected results. Third, changes in metabolism may not necessarily be reflected equally in the type of sample analyzed (i.e. if tamoxifen increases in serum, should it also in urine?).

Despite this, the question is raised as to which data (serum or urine) should be used to monitor metabolite levels. Table 3.2 and Table 4.2 listed some statistics of p-values obtained from paired t-tests for urine and serum respectively. Table 4.6 uses the same data from these tables, but makes a more direct comparison by including only results for tamoxifen, N–desmethyltamoxifen, and 4–hydroxytamoxifen, the compounds analyzed in both sample types. P-values appear to be larger in the tests with serum samples than urine samples. Using the differences of means test, the p-value is actually slightly higher in urine than serum. However, the standard deviation in urine is also much higher. Using the same data, the median is higher in serum than urine. Because p-values are larger for serum samples, there is less probability that there were significant differences with metabolite concentrations over time for serum than urine. Presumably, one would then use serum over urine for metabolic profiling.

Table 4.6. Statistics of p-values from paired t-tests of urine and serum samples. These tests were used to determine significant correlations between metabolic profiles from the same patients over a two-week period. Metabolites that were analyzed were N–desmethyltamoxifen, tamoxifen, and 4–hydroxytamoxifen.

	Urine: Compare means directly	**Serum: Compare means directly**	**Urine: Differences of means**	**Serum: Differences of means**
Number of tests (8 patients/urine test; 34 patients/serum test)	9	3	9	3
Mean	0.38	0.62	0.46	0.44
Standard deviation	0.18	0.11	0.30	0.13
Median	0.29	0.60	0.30	0.47
Minimum	0.21	0.52	0.12	0.30
Maximum	0.68	0.73	0.96	0.56

4.4 Discussion

It has been demonstrated that tamoxifen metabolic profiles in serum can be measured rapidly, reliably, and reproducibly with nonaqueous CE/ESI/MS.

Although many explanations have been proposed for tamoxifen resistance in patients, the mechanisms for clinical resistance to tamoxifen are still not yet fully established (14-28). Among the leading explanations for acquired tamoxifen resistance is variability in tamoxifen metabolism among patients and within breast tumors. Osborne *et al.* have demonstrated a wide range of intertumoral tamoxifen levels and accumulation of the less antiestrogenic E isomer of 4–hydroxytamoxifen in some patients on tamoxifen therapy (17,18) and this has been proposed to be a mechanism of acquired tamoxifen resistance. Other studies have failed to demonstrate a relationship between serum levels of tamoxifen and its major metabolites and *de novo* tamoxifen resistance (29,30).

Levels of tamoxifen and its metabolites have been measured in tumors or blood previously (18,31-35). One of these studies showed inconclusive results relating intratumoral tamoxifen metabolic profile and disease progression (18). Another study of 51 patients showed tamoxifen intratumoral concentrations were significantly lower in patients with acquired tamoxifen resistance. The concentrations in serum however for the same group of patients showed no significance differences (34). Yet another recent study showed significant accumulation of 4–hydroxytamoxifen in tumor tissues of patients with acquired tamoxifen resistance and significantly lower levels of 4–hydroxytamoxifen and N–desmethyltamoxifen in plasma of patients responding to tamoxifen treatment compared to non-responding patients. In this study, only six patients were counted in the group of non-responding patients (35). In this study here of tamoxifen metabolism, a previously inconclusive relationship has been identified between the tamoxifen metabolism profile and patient stage, which was independent of patient age, weight, height, and duration of tamoxifen usage. Those patients with early stage breast cancer had substantially higher serum levels of both tamoxifen and its metabolite, N–desmethyltamoxifen. The early stage breast cancer patients in this study had uniformly received standard surgical resection of all known disease (either by mastectomy or breast conserving surgery) prior to initiating adjuvant tamoxifen to reduce their risk of disease recurrence. In marked contrast, the advanced patients (stages III and IV) had surgically unresectable disease, and therefore had clinically evident cancer at the time of serum sampling. The most probable explanation for this observation is that the presence of clinically detectable breast cancer deposits influence tamoxifen metabolism. One possible mechanism for this is intratumoral metabolism of tamoxifen reducing the serum levels of tamoxifen and N–desmethyltamoxifen. It is unlikely that this effect is mediated by the enzymatic action of CYP 3A4, which converts tamoxifen to N–desmethyltamoxifen within hepatocytes (36,37), because this enzymatic activity has not been reported in cancer cells or non-liver tissue.

The proposed mechanism of intratumoral metabolism of tamoxifen reducing serum levels of tamoxifen and N-desmethyltamoxifen is supported by the analysis of urine samples from breast cancer patients undergoing tamoxifen treatment (Chapter 3). In that study, it was noted that advanced breast cancer patients had different urinary metabolite profiles than did those patients with early stage disease. Alternatively, macroscopic breast cancer might alter tamoxifen and N–desmethyltamoxifen metabolism by means of paraneoplastic effects on renal and hepatic function; however, clinically apparent paraneoplastic renal and hepatic complications are only rarely reported with breast cancer.

Concomitant drug use does not explain this finding, as no patient was taking medication known to alter tamoxifen metabolism. Although advanced stage patients in this study had a higher opioid analgesic usage than patients with early stage disease, these drugs have not been implicated in alterations of tamoxifen metabolism or of CYP 3A4 activity (38,39). It has been reported that ritonavir, erythromycin, cyclosporin, nifedipine, and diltiazem competitively inhibit N-demethylation of tamoxifen (36,38). Aminoglutehimide (40,41) and letrozole (42,43), aromatase enzyme inhibiting drugs to which none of the study subjects had been exposed, have also been shown to alter tamoxifen metabolism.

An individual's tamoxifen metabolic profile may contribute to variability in the efficacy of this drug. Some tamoxifen metabolites have an increased affinity for the estrogen receptor alpha, including 4–hydroxytamoxifen and 4–hydroxy–N–desmethyltamoxifen. In fact, 4–hydroxytamoxifen has been reported to be 100 times more potent than tamoxifen in inhibiting tumor growth (44,45). The affinity of 4–hydroxytamoxifen for ER-α is at least as great as that for estrogen. Also similar to estrogen, 4–hydroxytamoxifen shows a very slow dissociation rate from ER-α, which is unlike tamoxifen (46,47). Despite these similarities of 4–hydroxytamoxifen to estrogen, studies have shown that the metabolism of tamoxifen to 4–hydroxytamoxifen may be helpful, but not necessary, for the inhibition of estrogen action to occur (46,48). 4–Hydroxytamoxifen has agonistic properties in the breast. 4–Hydroxy–N–desmethyltamoxifen is similar to 4–hydroxytamoxifen in that it has a high affinity for the estrogen receptor and also has agonistic properties in the breast (44). N–Desmethyltamoxifen and N–didesmethyltamoxifen have a decreased affinity for the

ER. N–Desmethyltamoxifen also has a lower antagonistic activity than tamoxifen (49,50).

The finding that increased patient age was associated with higher levels of tamoxifen and N-desmethyltamoxifen was not unexpected, as this has previously been reported (51,52). The efficiency of clearance of many drugs declines with advancing age, due to age-related decline in renal and hepatic function.

4.5 Conclusions

Tamoxifen and several of its metabolites have been extracted from serum samples from 80 patients with all stages of breast cancer. Analyses of these metabolites were performed by nonaqueous CE/ESI/MS. Through these analyses it has been demonstrated that tamoxifen metabolic profiles in serum can be measured rapidly, reliably, and reproducibly with nonaqueous CE/ESI/MS. Serum tamoxifen and N–desmethyltamoxifen levels were lower in those patients with advanced breast cancer, suggesting that patients with large tumour burdens have altered tamoxifen metabolism compared to patients with early stage disease.

4.6 References

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4.7 Appendices

Appendix IV. Corrected signal (Pk Ht/Int. Std. Pk Ht) data for 34 patients used to determine changes in serum metabolic profiles over time.

Patient	Collection Date	N-didesmethyl tamoxifen	N-desmethyltamoxifen	tamoxifen	N-desmethyl-4-hydroxytamoxifen	4-hydroxytamoxifen
E040207	2/23/00	0.23239572	2.6349325	3.36035346	0.27134616	0.14570876
	3/8/00	0.30996408	2.97214227	2.42456708	0.3971699	0.12708976
E056651	4/28/00	0.06555716	0.87453957	0.43352763	0.03836041	0.02163551
	5/12/00	0.0703545	0.90674048	0.69591148	0.04919437	0.01907102
E085625	3/6/00	0.23487539	3.38761314	2.32940293	0.40175346	0.14696899
	3/20/00	0.20471452	2.64781275	1.6904896	0.31186766	0.07139788
E107381	2/8/00	0.10061094	1.96728754	1.75499429	0.27751453	0.0913078
	2/22/00	0.10204726	1.58879833	2.12864262	0.31020396	0.12702339
E108201	5/8/00	0.11862499	1.0513508	0.73327675	0.09151252	0.02316338
	5/22/00	0.10101641	1.00876464	0.74289267	0.09253054	0.02462129
E126494	2/16/00	0.08867319	1.97855131	2.00431205	0.2014169	0.09950074
	3/1/00	0.1423247	1.67686674	1.5745511	0.17808351	0.0809551

E147796	7/26/00	0.19979948	2.59056926	1.71011637	0.16698379	0.06325968
	8/9/00	0.13052112	1.57360983	0.88120679	0.1054214	0.03865754
E148422	1/17/00	0.0651487	0.94583643	1.22069703	0.121171	0.05322955
	1/31/00	0.10685167	0.9202138	0.86728235	0.11116334	0.03683157
E155490	6/22/00	0.139913	1.29439473	0.64600904	0.11937693	0.03894861
	7/6/00	0.16069957	2.34703835	1.61046875	0.24094815	0.09537997
E195611	5/19/00	0.08790001	1.97621782	2.60015571	0.09450872	0.09749602
	6/2/00	0.11919342	2.10474685	1.96241926	0.11234492	0.05375582
E203481	1/10/00	0.03677518	0.81622815	1.16837003	0.08454097	0.03836802
	1/24/00	0.07150588	1.47854875	1.20995375	0.14976568	0.06901463
E211521	5/1/00	0.06967965	1.3405986	0.63102054	0.05337011	0
	5/15/00	0.057417	1.34207523	0.89914984	0.06335572	0.03391439
E213876	1/17/00	0.1149257	1.57839476	1.55447597	0.14998497	0.07273749
	1/31/00	0.16537048	1.95831113	2.06948848	0.16447323	0.07587158
E217589	5/4/00	0.17678565	1.20965078	0.79281111	0.13987266	0.051175
	5/18/00	0.23718627	1.55402479	0.84098547	0.28473336	0.05606101
E219572	3/13/00	0.25115346	2.02148064	1.41657878	0.23626069	0.0793215
	3/29/00	0.13537227	1.56883429	0.77588702	0.1443269	0.03488259
E220454	2/23/00	0.0870267	1.22311687	1.16322773	0.16368688	0.06093999
	3/8/00	0.30500321	2.76776855	1.71158822	0.44032726	0.09686849
E225954	11/29/99	0.09095998	0.91237369	1.77722905	0.15545869	0.07387557
	12/13/99	0.19149491	2.28434934	1.99343757	0.38938224	0.11029758

E228354	1/10/00	0.07750606	1.5718492	1.3060315	0.13028236	0.04537513
	1/24/00	0.0622297	1.19379691	0.9200894	0.12709729	0.03640945
E229268	11/30/99	0.11517197	1.28817757	1.57690617	0.10405845	0.06635297
	12/14/99	0.09952988	1.32489006	1.11018919	0.10059495	0.06957042
E234321	3/27/00	0.40628805	3.73899027	2.38299665	0.29797571	0.06885249
	4/10/00	0.26289459	2.75305394	1.18383607	0.19928642	0.06958974
E236379	6/6/00	0.13728123	0.83909514	0.46360599	0.06813138	0.01775517
	6/20/00	0.15621939	1.28442601	1.07307157	0.10755752	0.04047278
E239330	2/2/00	0.08244547	1.23762938	1.66709549	0.14229577	0.07810654
	2/16/00	0.15409499	2.49059293	2.79695448	0.23011575	0.11735749
E241574	1/17/00	0.11335624	2.39153126	2.89657643	0.1525977	0.1520853
	1/31/00	0.19274187	3.74171597	2.94967938	0.27010999	0.13763439
E243242	3/6/00	0.51031956	3.66182448	2.40558787	0.64765157	0.13074966
	3/20/00	0.18980071	1.93836644	1.64896172	0.37459837	0.10006761
E244639	2/8/00	0.15805153	1.99055671	1.79543021	0.2060593	0.10559848
	2/22/00	0.14710628	2.31875425	2.09999826	0.23571017	0.14160315
E245526	1/17/00	0.0733242	1.40378706	1.09369431	0.09919183	0.04426554
	1/31/00	0.07501964	1.19543339	0.88027382	0.10731987	0.05747552
E246561	3/30/00	0.2354283	2.37260336	1.0913316	0.17682883	0.05572848
	4/14/00	0.21485159	1.90481355	1.10280091	0.12180601	0.05922675
E247773	7/10/00	0.16955597	2.8193621	2.62654841	0.19431008	0.09568025
	7/24/00	0.11967457	2.19794477	1.90835082	0.11313759	0.06798879

E250176	4/24/00	0.17070134	2.86176073	2.25082634	0.20127732	0.06453199
	5/8/00	0.09053166	1.36844731	1.09046714	0.09092872	0.04589988
E250759	8/8/00	0.08666747	0.97673163	1.0298055	0.1072238	0.03920936
	8/22/00	0.11183379	0.9768213	0.92610305	0.13634802	0.034451
E260600	4/3/00	0.24105423	2.6087875	1.36346683	0.25187173	0.07742912
	4/17/00	0.1545629	1.54782399	0.97347989	0.18246454	0.05607871
E260628	4/3/00	0.2214218	1.93487611	1.93709113	0.20154106	0.09740358
	4/17/00	0.37029691	2.57217021	2.19056804	0.20325671	0.11288899
E260880	7/10/00	0.157467	2.50507613	2.07500144	0.17167494	0.10266047
	7/24/00	0.16055128	3.62840839	2.9649727	0.30206511	0.14681322
E261100	2/28/00	0.20338041	1.6667007	1.72560263	0.24859622	0.08633089
	3/13/00	0.2580828	1.89187564	1.97220765	0.27519257	0.12303939

Appendix V. Patient clinical characteristics for serum analyses.

Patient	Initial Diagnosis	Tamoxifen Prescription	Cancer Stage	Date of Advanced/ Metastatic disease	Progression Date	Age (years)	Height (cm)	Weight (kg)
C014569	9/24/97	5/20/98	1	n/a	n/a	77	156	48
E040207	11/15/89	5/18/99	4	4/16/99	none	66	?	92
E049310	8/1/97	9/19/97	2	n/a	n/a	76	?	60
E056651	4/16/99	11/4/99	2	n/a	n/a	62	172	125
E078842	11/23/98	2/2/99	1	n/a	n/a	70	?	95
E081581	1/29/98	3/27/98	2	n/a	n/a	65	171	79
E085625	3/19/97	11/27/97	2	n/a	n/a	47	166	86
E106285	5/19/99	7/14/99	2	n/a	n/a	59	147	84
E107381	4/30/97	5/12/98	4	at diagnosis	3/7/00	53	173	78
E108201	11/6/95	9/19/96	2	n/a	n/a	58	157	53
E122138	8/6/98	11/10/98	2	n/a	n/a	63	165	108
E126494	?	4/20/98	2	n/a	n/a	57	162	66
E134580	10/9/97	12/10/97	2	n/a	n/a	58	162	67
E147796	1/4/96	10/9/96	3	n/a	n/a	57	151	90
E148422	12/11/98	3/5/99	2	n/a	n/a	62	175	105
E155490	1/25/99	9/24/99	2	n/a	n/a	47	163	60
E160884	7/17/87	4/10/00	4	3/23/00	none	64	155	61

E173485	6/13/89	12/16/99	4	11/4/99	none	73	?	61
E187831	11/23/99	1/27/00	1	n/a	n/a	73	158	95
E195611	1/23/91	12/1/97	4	4/9/97	none	58	154	77
E203481	9/28/98	1/18/99	1	n/a	n/a	46	158	56
E208317	7/29/93	2/3/99	4	12/29/98	none	56	163	102
E211485	10/14/97	6/29/98	4	7/29/98	none	93	?	48
E211521	3/30/94	7/6/99	4	6/4/99	none	41	166	70
E213876	7/22/99	1/16/99	2	n/a	n/a	75	164	78
E215670	7/17/95	12/1/95	2	n/a	n/a	48	167	76
E217589	5/19/95	8/29/95	2	n/a	n/a	74	170	71
E219572	10/30/95	10/23/98	4	8/13/98	none	54	155	115
E220454	6/27/94	8/10/99	4	7/9/99	none	50	172	74
E225954	5/28/96	7/2/96	2	n/a	n/a	44	148	56
E227157	?	?	4	2/8/00	?	42	156	65
E228354	4/16/97	12/8/97	2	n/a	n/a	46	155	65
E229268	8/27/97	8/17/99	4	8/5/99	none	73	?	74
E233123	6/19/96	2/23/99	4	12/9/98	9/28/00	57	160	83
E234321	9/27/96	10/23/96	2	n/a	n/a	59	?	68
E235829	12/23/96	4/14/97	2	n/a	n/a	55	151	54
E236379	2/7/97	9/9/97	3	n/a	n/a	53	164	94
E236972	3/14/97	12/11/97	2	n/a	n/a	55	159	80
E239330	8/13/97	2/18/98	2	n/a	n/a	43	163	119
E239840	10/7/97	1/22/98	1	n/a	n/a	62	154	73

E240236	10/8/97	11/10/97	3	n/a	n/a	47	162	87
E240518	10/20/97	5/12/98	2	n/a	n/a	39	148	53
E241574	1/17/97	1/11/99	2	n/a	n/a	45	167	68
E243107	3/25/98	10/19/98	2	n/a	n/a	52	161	59
E243242	3/31/98	4/20/98	2	n/a	n/a	45	169	71
E243290	4/3/98	6/1/98	1	n/a	n/a	82	158	77
E243834	5/11/98	12/8/98	3	n/a	n/a	65	156	101
E244015	5/19/98	2/12/98	2	n/a	n/a	48	161	71
E244639	?	9/22/98	2	n/a	n/a	60	161	84
E245526	8/14/98	9/23/98	2	n/a	n/a	75	160	56
E246561	10/15/98	7/8/99	2	n/a	n/a	49	166	74
E246639	10/20/98	11/20/98	1	n/a	n/a	54	162	120
E246673	10/21/98	5/6/99	2	n/a	n/a	50	154	73
E247773	12/17/98	8/26/99	2	n/a	n/a	43	158	86
E248172	1/25/99	6/6/99	2	12/4/00	12/27/00	46	168	77
E248577	1/29/99	4/19/99	2	n/a	n/a	65	172	81
E249883	4/7/99	12/7/99	2	n/a	n/a	50	155	75
E250176	12/2/97	7/28/98	4	5/19/98	none	55	168	91
E250448	1/21/98	6/8/99	4	3/24/99	none	46	153	56
E250759	3/3/98	11/12/98	3	n/a	n/a	54	158	67
E252164	10/6/98	7/5/99	4	5/1/00	5/1/00	42	150	64
E252647	5/19/98	11/16/98	2	n/a	n/a	70	155	60
E254793	8/9/99	10/6/99	2	n/a	n/a	49	170	79

E260600	5/11/99	7/23/99	2	n/a	n/a	59	155	54
E260628	5/12/99	11/10/99	2	n/a	n/a	48	160	70
E260880	5/26/99	9/28/99	1	n/a	n/a	68	165	64
E261029	?	11/30/99	2	n/a	n/a	50	168	82
E261093	6/9/99	6/22/00	2	n/a	n/a	47	155	66
E261100	6/8/99	9/8/99	4	at diagnosis	6/19/00	71	156	92
E261108	6/8/99	6/12/00	2	n/a	n/a	58	161	89
E261531	6/28/99	3/17/00	2	n/a	n/a	62	161	58
E261555	6/25/99	2/8/00	2	n/a	n/a	52	166	68
E262293	8/18/99	9/13/00	2	n/a	n/a	57	164	107
E262309	8/19/99	9/19/00	2	n/a	n/a	51	163	57
E262333	8/20/99	3/1/00	2	n/a	n/a	47	153	67
E262659	9/3/99	3/28/00	2	n/a	n/a	61	164	69
E263697	11/17/99	12/29/99	1	n/a	n/a	55	?	94
E264724	12/20/99	4/25/00	4	5/26/00	none	57	163	93
E265332	1/10/00	7/20/00	2	n/a	n/a	48	165	84
E265730	2/9/00	3/15/00	1	n/a	n/a	61	?	71
G001064	11/26/99	6/6/00	1	n/a	n/a	49	163	51

Appendix VI. Corrected signal (Pk Ht/Int. Std. Pk Ht) data used to determine associations between patient clinical characteristics (see Appendix V) and serum metabolic profiles.

Patient	N-didesmethyltamoxifen	N-desmethyltamoxifen	tamoxifen	N-desmethyl-4-hydroxytamoxifen	4-hydroxytamoxifen
C014569	1.032078562	11.27265385	5.531543826	0.862444855	0.116115592
E040207	0.309964077	2.972142265	2.42456708	0.397169898	0.127089762
E049310	0.553498026	9.100290993	2.496204876	0.407726588	0.082607545
E056651	0.070354505	0.906740478	0.695911478	0.049194366	0.019071019
E078842	0.35135939	5.404108379	2.437347214	0.333187712	0.048259516
E081581	0.197695891	3.996680521	1.860860833	0.181321655	0.047398812
E085625	0.204714521	2.647812754	1.690489601	0.31186766	0.071397876
E106285	0.061674732	0.91368702	1.234536536	0.092713611	0.030710488
E107381	0.102047258	1.588798333	2.12864262	0.310203956	0.127023393
E108201	0.101016409	1.008764643	0.742892667	0.09253054	0.024621286
E122138	0.252692121	4.466138409	2.222888941	0.235033052	0.026248957
E126494	0.1423247	1.676866738	1.574551095	0.178083511	0.080955095
E134580	0.225839917	4.391501803	3.07474554	0.259661494	0.054265422
E147796	0.130521115	1.573609825	0.881206794	0.1054214	0.038657542
E148422	0.106851669	0.920213801	0.86728235	0.111163341	0.036831569

E155490	0.160699574	2.347038352	1.61046875	0.240948153	0.095379972
E160884	0.171075943	2.767090153	2.125141137	0.214224735	0.063536723
E173485	0.07857771	1.414709503	1.185343261	0.193943835	0
E187831	0.258080029	6.498437036	3.724465693	0.284402891	0.07964752
E195611	0.119193417	2.104746847	1.962419258	0.112344915	0.053755821
E203481	0.071505876	1.478548748	1.209953749	0.14976568	0.069014631
E208317	0.271920533	3.403737966	1.736841796	0.267948356	0
E211485	0.714139357	8.625516746	2.879467977	0.348936553	0.03271224
E211521	0.057416996	1.342075229	0.899149838	0.063355719	0.033914391
E213876	0.165370476	1.958311128	2.069488479	0.164473229	0.075871579
E215670	0.18984871	3.31707868	1.381962143	0.175183031	0
E217589	0.237186274	1.554024786	0.840985472	0.284733358	0.056061012
E219572	0.135372274	1.568834294	0.775887023	0.144326899	0.034882594
E220454	0.30500321	2.767768547	1.711588225	0.440327256	0.096868494
E225954	0.191494908	2.284349339	1.993437569	0.389382236	0.110297576
E228354	0.062229696	1.193796909	0.920089398	0.127097291	0.036409449
E229268	0.099529879	1.324890057	1.110189194	0.100594952	0.069570421
E233123	0.279729582	3.226376759	2.118779463	0.227875023	0.033371094
E234321	0.262894591	2.753053936	1.183836066	0.199286421	0.069589745
E235829	0.055248223	1.237206993	0.495568209	0.112945029	0
E236379	0.156219389	1.284426011	1.073071575	0.107557522	0.040472782
E236972	0.185936855	3.972185112	1.729184267	0.190853455	0.090836755
E239330	0.154094994	2.490592929	2.796954481	0.230115754	0.117357487

E239840	0.212656714	5.213728967	1.667683933	0.247020373	0
E240236	0.193142428	2.907147292	1.594281908	0.192269388	0.029114284
E240518	0.348806986	4.527890541	3.83943226	0.465874244	0
E241574	0.192741874	3.74171597	2.949679383	0.270109995	0.137634394
E243107	0.41459773	5.014904272	2.048148197	0.338120846	0
E243242	0.189800715	1.93836644	1.64896172	0.374598371	0.100067609
E243290	0.436457738	7.26446886	3.064515093	0.515857472	0.076991736
E243834	0.099224614	2.256811353	0.51156889	0.1206687	0
E244015	0.403481759	3.856688947	1.972800983	0.297711758	0
E244639	0.147106278	2.318754252	2.099998256	0.235710174	0.141603147
E245526	0.075019645	1.19543339	0.880273815	0.107319874	0.05747552
E246561	0.214851594	1.90481355	1.102800914	0.121806006	0.059226748
E246639	0.142159138	5.6702465	1.988830528	0.163994821	0.046896147
E246673	0.206413614	3.51327914	1.781033289	0.273352152	0
E247773	0.119674571	2.19794477	1.90835082	0.113137592	0.067988788
E248172	0.144088422	3.73016702	1.986820681	0.159619605	0.03515509
E248577	0.226204314	3.105876275	1.627457058	0.300180808	0
E249883	0.448741278	6.891352789	2.916465554	0.234870273	0.030587147
E250176	0.09053166	1.368447305	1.090467139	0.090928718	0.045899882
E250448	0.296374973	3.524673069	1.269952186	0.259036481	0.048347365
E250759	0.111833785	0.976821298	0.926103052	0.136348017	0.034450998
E252164	0.037654329	1.161907182	0.517368086	0.060558645	0
E252647	0.141559706	6.291887122	4.216712052	0.20945928	0.118949238

E254793	0.053805682	1.42482337	1.578203137	0.074089292	0.045427669
E260600	0.154562905	1.547823991	0.973479893	0.182464537	0.056078708
E260628	0.370296912	2.572170214	2.19056804	0.203256709	0.11288899
E260880	0.160551283	3.628408387	2.964972696	0.302065111	0.146813216
E261029	0.144595144	2.219785634	1.272371228	0.132672966	0.029579397
E261093	0.181244478	2.517132159	1.507888426	0.14959461	0.019364959
E261100	0.258082803	1.891875639	1.972207646	0.275192566	0.123039387
E261108	0.323986214	3.869925616	2.888009074	0.284110988	0.059660144
E261531	0.490089127	9.987593583	4.442875817	0.692442068	0.136482472
E261555	0.267629027	6.316040575	1.871686546	0.223353026	0.06075131
E262293	0.345290172	6.664339965	3.600162299	0.426071741	0.054465112
E262309	0.153709948	2.257968064	1.646160697	0.130277583	0.044802468
E262333	0.104305342	2.050818547	1.279889859	0.109280385	0.028002388
E262659	0.127707563	2.86186468	2.195308066	0.282962804	0.070278853
E263697	0.21775974	1.346977232	1.830609014	0.214994536	0.07280958
E264724	0.22854641	3.893395257	1.708916181	0.223015363	0.044924516
E265332	0.402201768	3.850824884	3.543931461	0.319363641	0.059999723
E265730	0.060921007	1.483292404	1.021862102	0.08970006	0.036975721
G001064	0.164657229	3.525560951	1.604121556	0.23192631	0.060958229

Chapter 5

Two In–Vitro Tamoxifen Experiments

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5.1 Experiment One: Nonaqueous Capillary Electrophoresis of Tamoxifen and Its Acid Hydrolysis Products

5.1.1 Introduction

Most tamoxifen metabolites are not commercially available. Acid hydrolysis is a convenient method of generating tamoxifen degradation products. A simple procedure is employed in this study to produce hydroxylated tamoxifen metabolites by incubating tamoxifen with 0.1 M hydrochloric acid. Not all of these hydroxylated products may be produced in vivo. The compounds are referred to as degradation products to distinguish them from the product of metabolic action.

The purposes for this in vitro study are: First, to mimic stomach–like conditions that tamoxifen encounters after it is administered orally. Once digested, tamoxifen settles in stomach acid at a high pH and degrades. Second, nonaqueous capillary electrophoresis (CE) is used with both thermo–optical absorbance detection (TOAD) and electrospray mass spectrometry (ESI/MS) detection to analyze these tamoxifen degradation products.

5.1.2 Experimental

5.1.2.1 Reagents

Tamoxifen was purchased from Sigma (St. Louis, MO, U.S.A.). A stock solution of tamoxifen (500 μg/mL) was prepared in 100% methanol and working solutions were prepared by dilution with methanol. Ammonium acetate and hydrochloric acid at analytical grade were obtained from ACP Chemicals (Montreal, PQ). HPLC–grade methanol and acetonitrile were purchased from BDH (Toronto, ON). Fused silica capillary was purchased from Polymicro Technologies (Phoenix, AZ, U.S.A.).

5.1.2.2 Instrumentation

The CE/TOAD system has been described in detail (1,2). Briefly, a modulated pump laser beam periodically illuminates the sample at a point near the exit of the capillary producing a temperature rise within a sample due to nonradiative relaxation following absorbance. The temperature rise is proportional to the laser power and the sample absorbance. This temperature rise is detected as a change in the refractive index of the heated sample (the refractive index for most liquids will change a few ppt for every 1°C increase). Complicated deflection and diffraction effects occur at the capillary–solution interface. Perturbation of the refractive index at the interface changes the intensity of the probe beam, measured after the capillary with a small photodiode. Phase–sensitive detection is used to demodulate the intensity change (1).

A 45 cm x 50 μm i.d. x 180 μm o.d. capillary with 39 cm to the detection window was used. The running buffer was composed of 20 mM ammonium acetate in 70% methanol / 30% acetonitrile for the separation. The applied voltage across the capillary was 12 kV. Sample injection was performed at 5 kV for 5 s.

The same CE/ESI/MS system used for experiments described in Chapters 2–4 is used here. The coaxial sheath flow liquid was 100% methanol and was introduced by a syringe pump (Harvard Apparatus, Southnatick, MA, U.S.A.) at a flow rate of 2.0 μL/min. Capillaries for both CE and sheath flow were 55 cm long with the same dimensions as those described above. CE separation was carried out with the applied voltage of 20 kV at the injection end. Positive ion mode was used for all measurements. Ion spray voltage was 3500 V, orifice voltage 10 V, ring voltage 260 V, nebulizer gas flow rate 0.67 L/min (NEB 1), curtain gas flow 0.95 L/min (CUR 8) and CEM voltage 2300 V. During sample injections, the ionspray voltage was set to 0 and the nebulizer gas flow rate was set to 0.03 L/min (NEB 0). Total ion electropherograms were acquired using scan mode (200 to 800 amu) at scan speed 1.21 s, step size 1.0 amu, and dwell time 1.5 ms. The mass spectrometer was operated under vacuum at 0.9×10^{-5} Torr.

5.1.2.3 Incubation of Tamoxifen in 0.1 M HCl and Sample Preparation

Acid–mediated tamoxifen degradation experiments were carried out by incubation in 0.1 M HCl. Tamoxifen solutions at 0, 1, 5, 10, and 12.5 μg/mL were prepared by adding appropriate amounts of 500 μg/mL stock methanol solution into 2 mL of 0.1 M HCl. Another set of tamoxifen solutions was prepared in water as a control. All of these solutions were kept in an incubator at 37 ^{O}C. Aliquots of 50 μL were periodically withdrawn from each solution and quickly dried using vacuum centrifugation. The dried residues were kept at 4 ^{O}C until analysis. For nonaqueous CE/ESI/MS analysis, the dried residue was redissolved in 50 μL of methanol.

5.1.3 Results and Discussion

Hydroxylated tamoxifen degradation products were generated by incubation of tamoxifen with 0.1 M hydrochloric acid. This acid mixture mimics stomach acidity and provides a useful source of degradation products, particularly those that are not commercially available. A set of seven degradation products was generated in addition to the parent compound. A typical nonaqueous CE separation of the tamoxifen samples detected by TOAD and ESI/MS is shown in Figures 5.1 and 5.2.

In Figure 5.1, tamoxifen and five degradation products were detected by nonaqueous CE/TOAD. In Figure 5.2, when the same sample was analyzed using nonaqueous CE/ESI/MS, tamoxifen and seven products were separated and detected. In Figure 5.1, Peaks 3 and 7 were not detected by TOAD. In Figure 5.2, Peaks 3, 6, and 7 were barely detected in the total ion electropherogram. Extracted ion electropherograms revealed the components.

Figure 5.1. Analysis of a 5 µg/mL tamoxifen extract sample after incubation with 0.1 M HCl at 37 °C for 72 days by CE/TOAD. The inset shows an expanded view of the TOAD data.

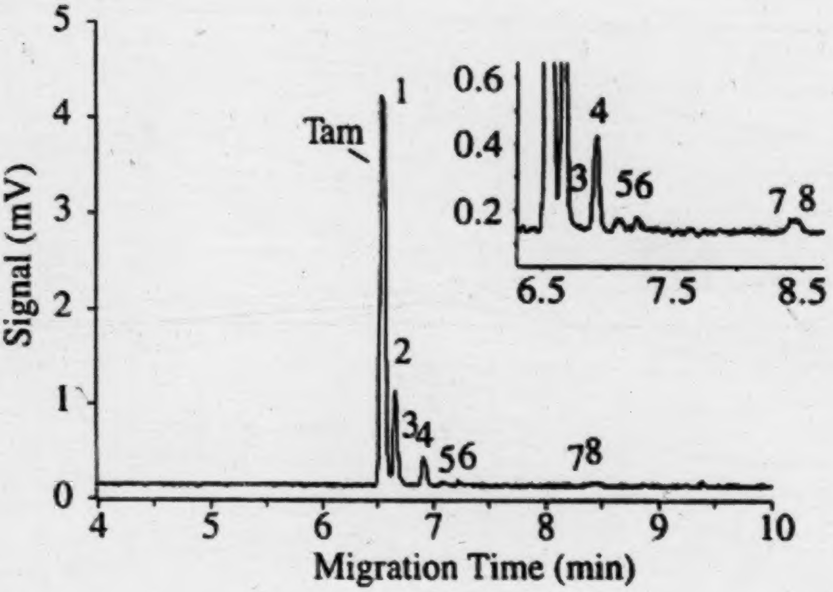
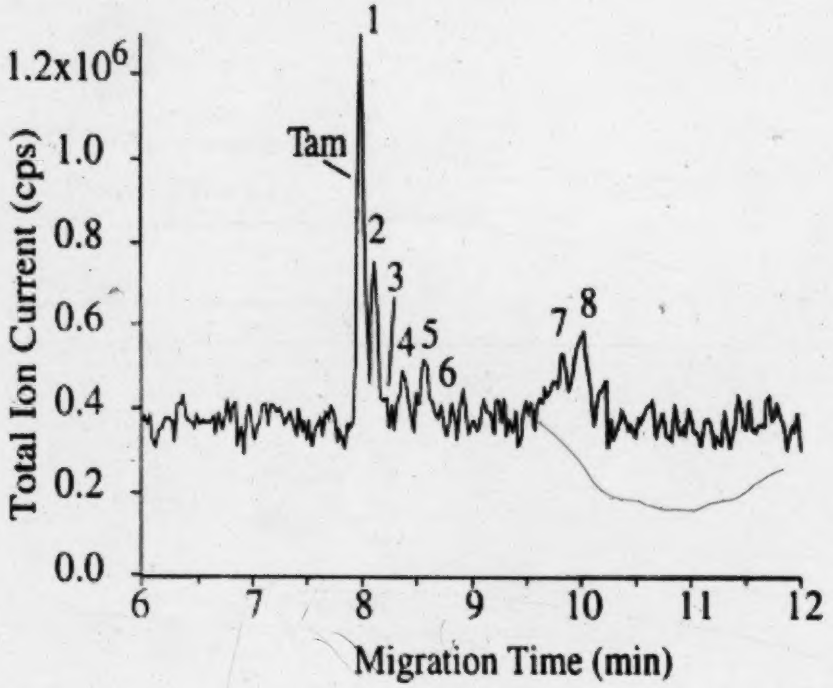


Figure 5.2. Total ion electropherogram of a 5 µg/mL tamoxifen extract sample (same sample used in Figure 5.1) by CE/ESI/MS, presented as a TIC trace.



The tamoxifen standard co–migrated with the first peak from the sample. Relative migration times of the acid degradation products from CE/TOAD matched those from CE/ESI/MS.

CE/TOAD was used to monitor the changes of tamoxifen in acid at 37 °C. Peak 2 slowly increased as incubation time progressed (data not shown). Peaks 3 and 7 were not detected. Peak 4 was detected only after seven days incubation.

The purity of the peaks was confirmed using CE/ESI/MS by examining the mass spectra across the entire peak. Figure 5.3 shows the ESI mass spectra of tamoxifen and the other product peaks. Figure 5.4 gives the product pathway. Peak 1 is the parent compound, tamoxifen. Peak 2 had an identical mass spectrum to that of tamoxifen, suggesting that it may be a structural isomer. The mass spectrum of Peak 3 suggests that it might be 4–hydroxytamoxifen. Peak 4 is tentatively identified as 3,4–dihydroxytamoxifen. Peak 5 did not give a useful spectrum to indicate whether this peak is a tamoxifen degradation product or a contaminant. The spectrum of Peak 6 was similar to that of Peak 4, suggesting that they are structural isomers of 3,4–dihydroxytamoxifen. Peaks 7 and 8 are tentatively identified as dimers of Metabolite E (M.W. 300) and N–desmethyltamoxifen (M.W. 357), respectively. Comparing intensities of peaks showed that the major acid–hydrolysis products were the tamoxifen isomer (possible E–form, Peak 2) and 3,4–dihydroxytamoxifen (Peak 4). This nonenzymatic process is different from the enzymatic transformation where the major components are tamoxifen and 4–hydroxytamoxifen in urine (3) and N–desmethyltamoxifen in serum (4).

Figure 5.3. Mass spectra of the eight peaks from Figure 5.2 detected by nonaqueous CE/ESI/MS.

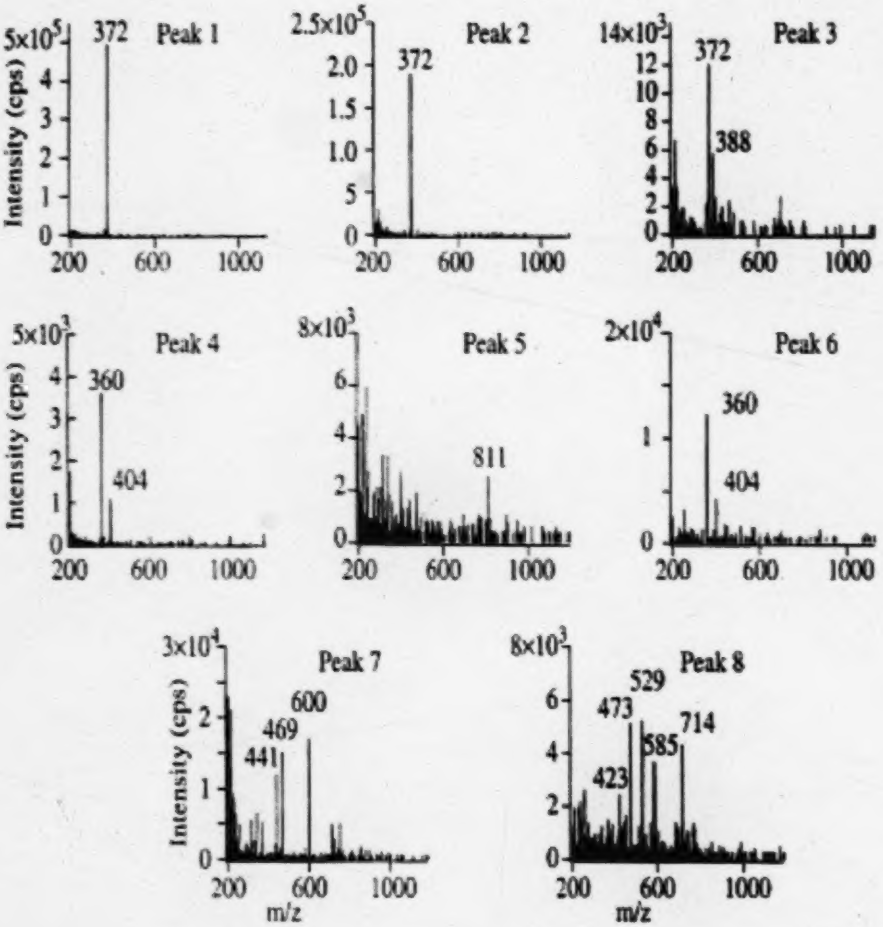
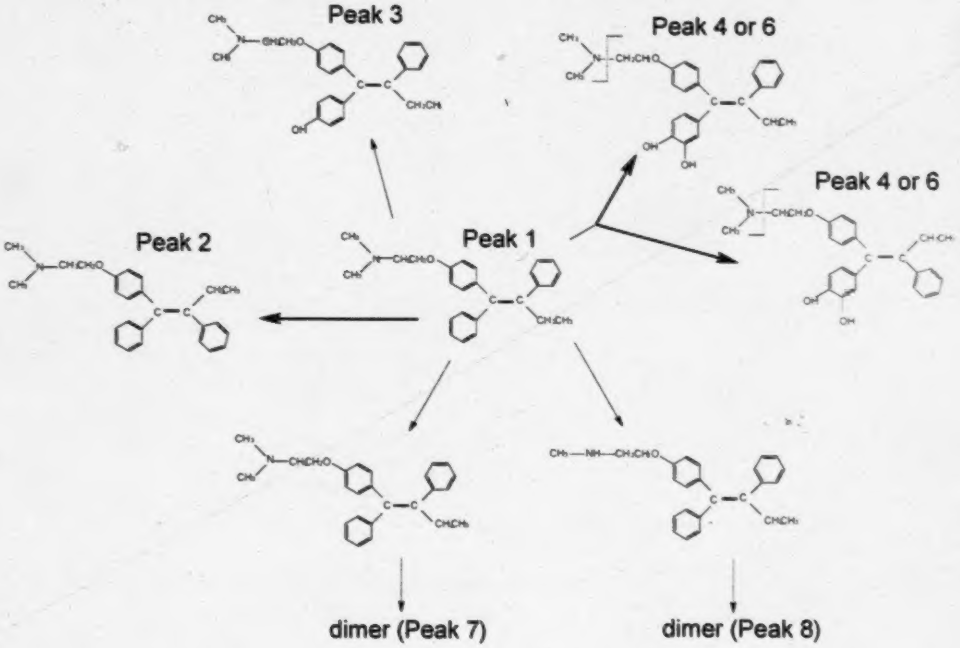


Figure 5.4. Product pathway for tamoxifen and its acid hydrolysis products. Peaks correspond to those in Figures 5.2 and 5.3. Arrows in bold show the major products.


Peak 3
Peak 4 or 6
Peak 4 or 6
Peak 2
Peak 1
dimer (Peak 7)
dimer (Peak 8)

5.1.4 Conclusions

Tamoxifen and its acid hydrolysis products were separated and tentatively identified by nonaqueous capillary electrophoresis with thermo–optical absorbance (CE/TOAD) and electrospray–ionization mass spectrometry (CE/ESI/MS). It has been demonstrated that nonaqueous CE can separate tamoxifen and some of its degradation products without the use of surfactants. Nonaqueous CE/ESI/MS gives both the migration time and a mass spectrum, which aids in the identification of the degradation products. Acid hydrolysis is a convenient method of generating tamoxifen degradation products.

5.2 Experiment Two: Analysis of Tamoxifen and its Metabolites in Synthetic Gastric Fluid Digests using High Performance Liquid Chromatography with Electrospray Mass Spectrometry

5.2.1 Introduction

Tamoxifen is administered orally. The fate of tamoxifen in gastric fluid has not previously been reported. In this study, nonenzymatic transformation of tamoxifen in synthetic gastric fluid and the effects of pepsin (a digestive enzyme) on this process are investigated. Tamoxifen transformation products in gastric fluid are analyzed. Also, the use of extracted ion chromatograms from HPLC–MS for the analysis of tamoxifen and its metabolites in a complex matrix to minimize sample preparation procedures is demonstrated.

5.2.2 Experimental

5.2.2.1 Reagents

Tamoxifen, pepsin, and synthetic gastric fluid were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). The E and Z isomers of 4–hydroxytamoxifen were obtained from Research Biochemical International (Natik, MA, U.S.A.). HPLC grade acetonitrile and methanol were obtained from EM Science (Gibbstown, NJ, U.S.A.). Analytical grade formic acid was purchased from BDH Inc. (Toronto, ON).

5.2.2.2 Instrumentation

The HPLC system consisted of a pair of pumps (Perkin Elmer Series 200 Micro, Norwalk, CT, U.S.A.) with a 75 μL dynamic mixer, a Keystone (Bellefonte, PA, U.S.A.) BDS Hypersil C18, 3 μm, 120Å, 50 mm x 1 mm column, and an autosampler (Perkin Elmer Series 200). The mobile phase was composed of solvent A (0.1% formic acid in deionized water) and solvent B (0.1% formic acid in acetonitrile). Separation was carried out by gradient elution from 5 to 95% of solvent B in 50 minutes at a flow rate of 60 μL/min. The injection volume of samples was 3 μL.

The mass spectrometers used were an API 3000 triple quadrupole system or a tandem quadrupole time-of-flight system (Model Qstar, PE–SCIEX Instruments, Concord, ON) connected to the HPLC using an ion spray source. The electrospray ionization conditions were ion spray voltage 5400 V, orifice voltage 65 V, ring voltage 220 V, curtain gas (CUR) 12, and nebulizer gas (NEB) 12. The full scan mass range was 250-810 amu with a step size of 0.25 amu and dwell time of 0.8 ms.

5.2.2.3 Tamoxifen Digestion in Synthetic Gastric Fluid

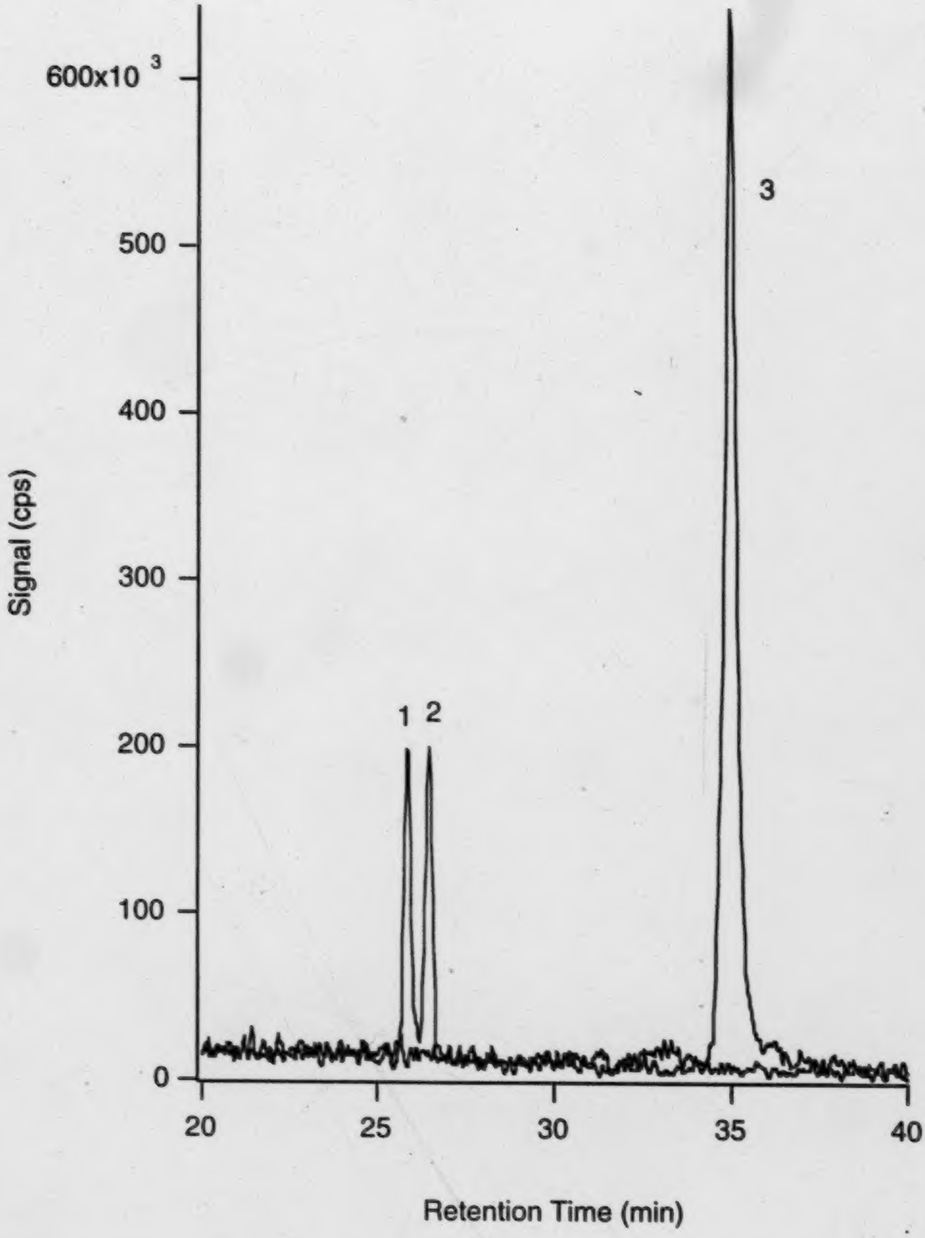
Gastric fluid-mediated digestion was conducted to simulate tamoxifen metabolism in the stomach. Concentrations of tamoxifen at 0, 1, 5 and 10 μg/mL were incubated with synthetic gastric fluid (mainly consisting of NaCl and HCl at pH 1.4) at 37°C for one week. Pepsin, a major digestive enzyme in the stomach, was added to a second set of samples to examine its effect on tamoxifen metabolism.

5.2.3 Results and Discussion

5.2.3.1 Method and Calibration

Three commercially available standards, (Z)–tamoxifen, (Z)– 4–hydroxytamoxifen, and (E)–4–hydroxytamoxifen, were separated and detected using HPLC/ESI/MS. Despite the structural similarity of these compounds, they were completely resolved as shown in Figure 5.5. Unlike separation of 4–hydroxytamoxifen isomers using nonaqueous CE (Section 2.3.2.1), in the reversed-phase separation of these standards shown in Figure 5.5, (E)–4–hydroxytamoxifen eluted before (Z)– 4–hydroxytamoxifen. Based on this information, one would predict that (E)–tamoxifen will elute before (Z)–tamoxifen in product samples using this analytical method. The reproducibility of retention times and detection limits using this method was also examined. The relative standard deviations (RSD, n=5) of retention times were 0.2% for (E)–4–hydroxytamoxifen (25.76 min), 0.2% for (Z)–4–hydroxytamoxifen (26.36 min), and 0.5% for (Z)–tamoxifen (34.69 min). The calibration curves (based on peak heights versus concentrations) of the three components from 50, 100, 500, and 1000 ng/mL samples were linear with $R^2 > 0.97$. The HPLC/ESI/MS method was applied to study nonenzymatic transformation of tamoxifen in a synthetic gastric fluid to mimic human stomach conditions.

Figure 5.5. Superimposed extracted ion chromatograms of m/z 388 and 372. Peak 1: (E)–4–hydroxytamoxifen (m/z 388). Peak 2: (Z)–4–hydroxytamoxifen (m/z 388). Peak 3: (Z)–tamoxifen (m/z 372).



5.2.3.2 HPLC/MS Analysis of Tamoxifen and its Transformation Products in Gastric Fluid without Extraction

Four analyses occurred where there was sequential injection into the HPLC/ESI/MS system without extraction. These four analyses were: (i) a tamoxifen digest in gastric fluid with pepsin, (ii) a tamoxifen digest in gastric fluid without pepsin, (iii) gastric fluid and pepsin without tamoxifen, as a control, and (iv) tamoxifen in methanol as a standard (data not shown). The total ion chromatograms from the samples are compared in Figure 5.6. A few product peaks in Figure 5.6 are difficult to observe. However, the product peaks are clearly observed when extracted ion chromatograms are compared.

Figure 5.7 shows the extracted ion chromatograms for tamoxifen (m/z 372.0) from the digest without pepsin, the digest with pepsin, and the control. Two chromatographic peaks are found in the digests and none in the control. Only one peak from the (Z)–tamoxifen standard solution (data not shown) was detected. These results suggest that the small peak eluting before (Z)–tamoxifen was produced in the gastric fluid. The two chromatographic peaks detected in the digests are probably due to acid–catalyzed isomerization of tamoxifen. Comparing the retention times of these two peaks with that of the (Z)–tamoxifen standard, the second peak was identified as (Z)–tamoxifen. The product peak was tentatively identified as (E)–tamoxifen, based on its mass spectrum and comparison with the elution order of the E and Z isomers of 4–hydroxytamoxifen standards (Figure 5.5). The ratios of peak intensity (E/Z isomers of tamoxifen) were 0.4 from the digests without pepsin and 0.1 with pepsin (Figure 5.7). Pepsin did not enhance the degradation process, but instead protected (Z)–tamoxifen from transforming into the (E)–isomer in acidic solution. This suggests that proteins in the human stomach may reduce nonenzymatic degradation of tamoxifen.

3,4–Dihydroxytamoxifen (m/z 404) is another potential product. The extracted ion chromatograms of m/z 404 are shown in Figure 5.8. There are three chromatographic

Figure 5.6. Total ion chromatograms from tamoxifen digest without pepsin, a digest with pepsin, and control with gastric fluid, pepsin, but not tamoxifen.

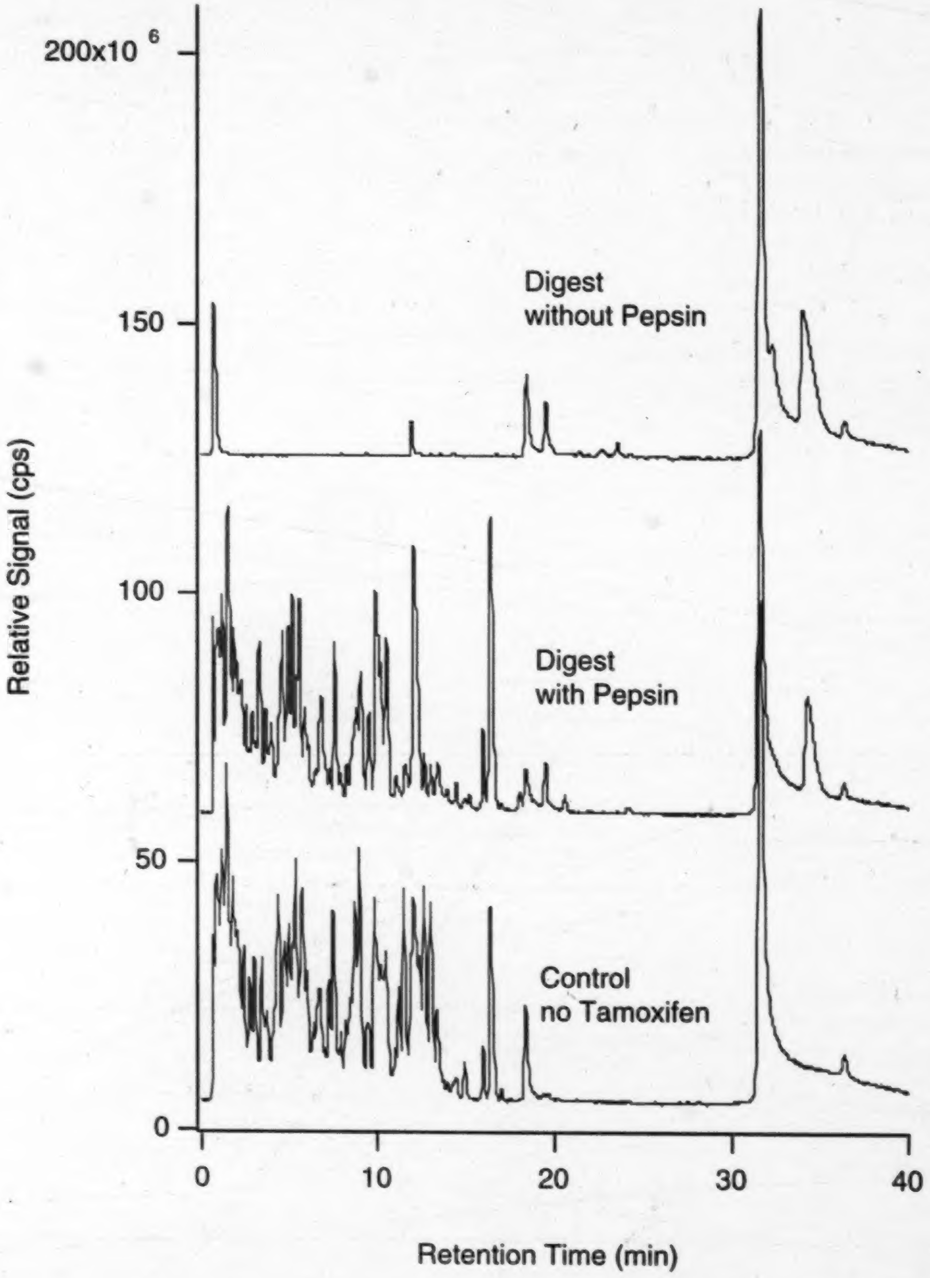


Figure 5.7. Extracted ion chromatograms of m/z 372 from the same samples shown in Figure 5.6.

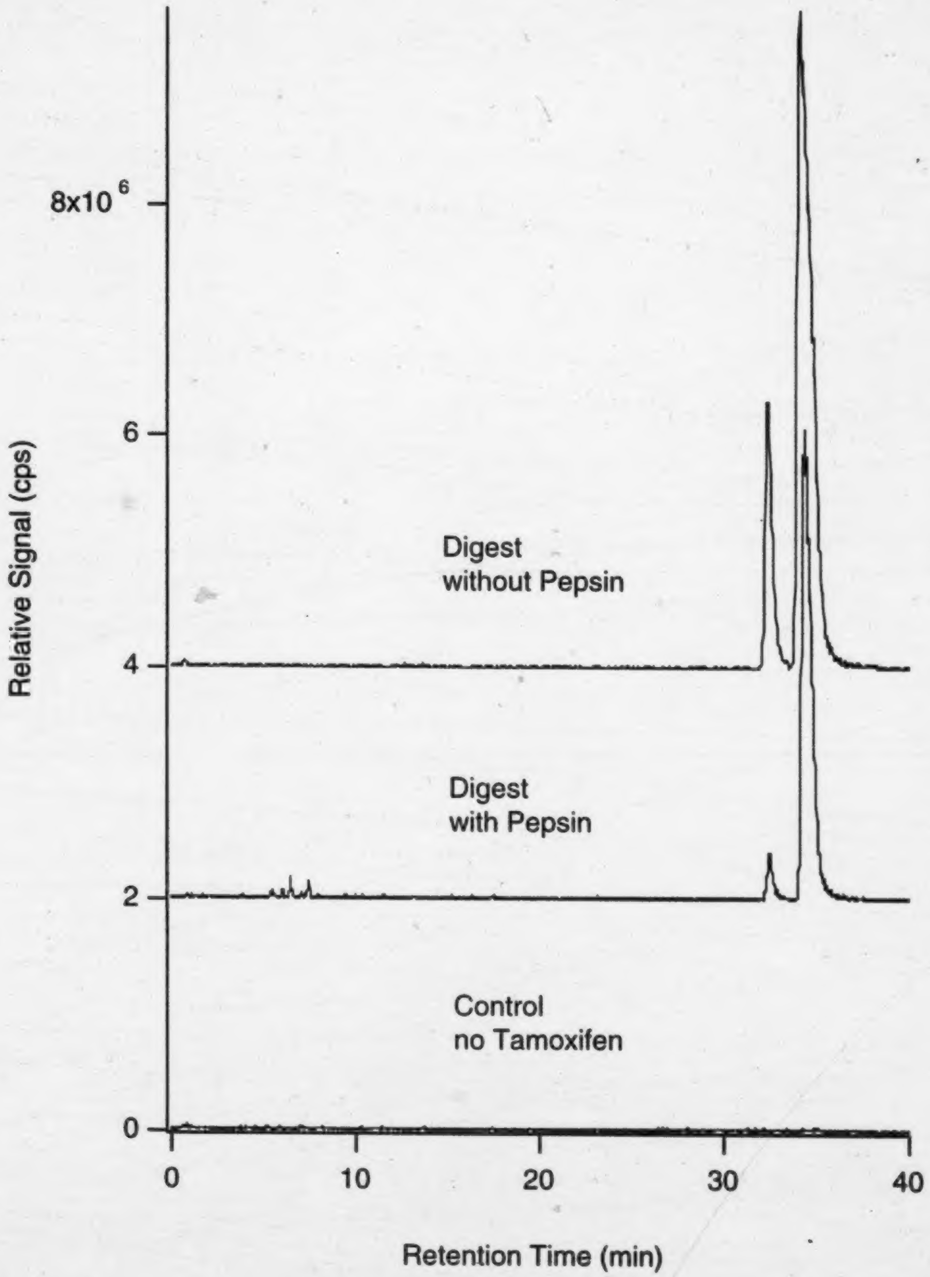
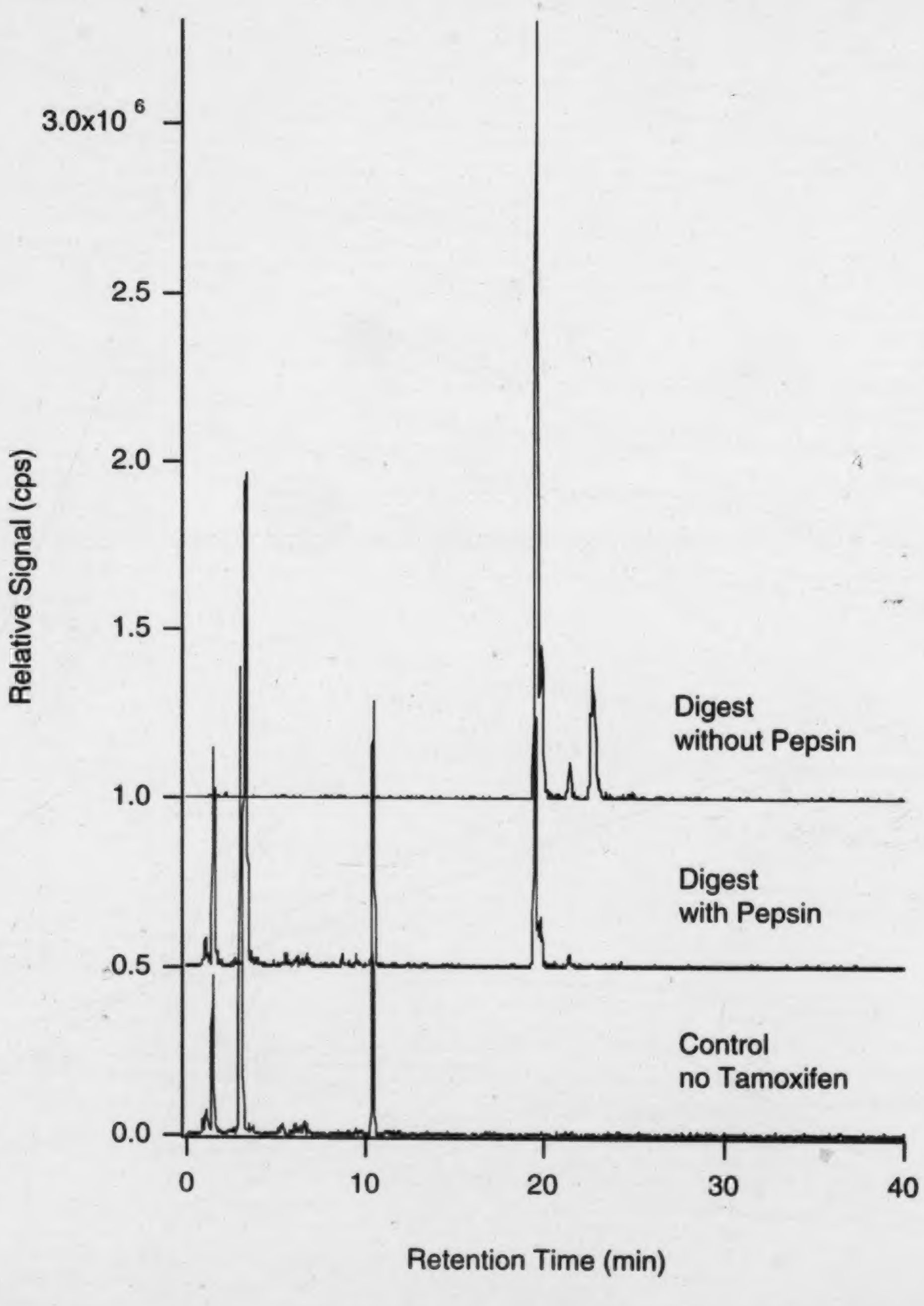


Figure 5.8. Extracted ion chromatograms of m/z 404 from the same samples shown in Figure 5.6.



peaks in the digests shown in Figure 5.8. The non-pepsin digest produced approximately 6 times more product than the digest with pepsin, based on the intensity of the main peak. This further supports the hypothesis that pepsin reduces the degradation rate of tamoxifen.

4–Hydroxytamoxifen (m/z 388) is commonly found in the biological samples from patients on tamoxifen treatment but sometimes at low concentrations. The presence of 4–hydroxytamoxifen in the gastric fluid digests was investigated but not detected in the samples described above. This suggests that 4–hydroxytamoxifen is primarily produced by enzymatic transformation in humans.

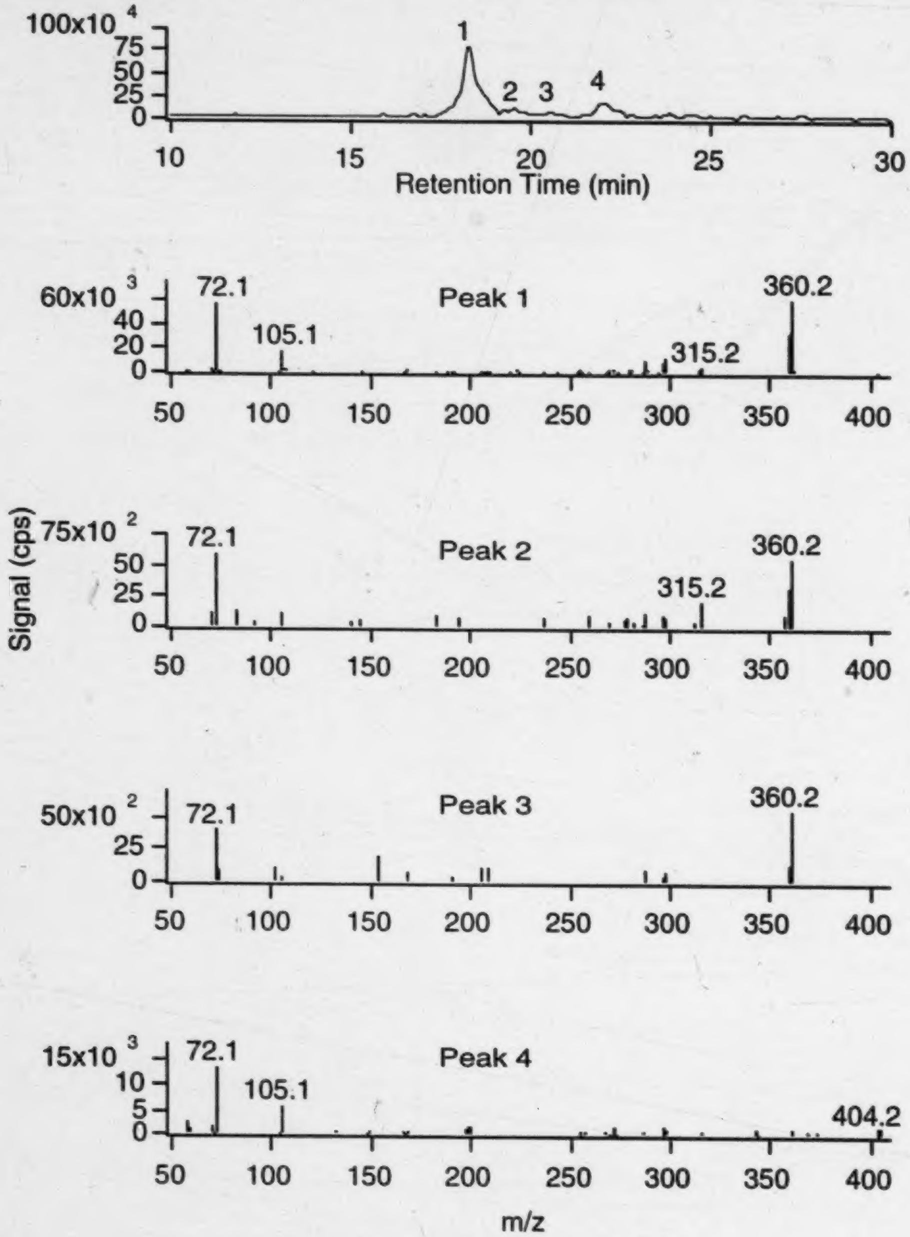
5.2.3.3 HPLC/MS/MS Analysis of the Major Products at m/z 372 and 404

The major transformation products were further analyzed at m/z 372 and 404 using HPLC/MS/MS. The two species at m/z 372 described above produced very similar product ion spectra consisting of major fragment ions of m/z 72 and 372. These similar spectra support the identification of the two chromatographic peaks at m/z 372 as the E and Z isomers of tamoxifen. Again, the E isomer of tamoxifen being the first to elute under these conditions and the Z isomer the second.

Figure 5.9 shows the chromatogram of m/z 404 obtained using HPLC/MS/MS and the product ion spectra of the corresponding peaks. Four chromatographic peaks corresponding to m/z 404 were detected at retention times of 18.4, 19.6, 20.6 and 22.0 min. Peaks 1 to 3 produced similar product ion spectra consisting of major fragments at m/z 360 and 72.

Unlike peaks 1 to 3, Peak 4 at 22.0 minutes showed a different MS/MS spectrum. This spectrum consisted of major ions at m/z 404, 105, and 72, but the fragment m/z 360 was absent. Mass ions of m/z 404 and 72 corresponds to $(M + H)^+$ of 3,4–dihydroxytamoxifen and $(CH_3)_2N(CH_2)_2^+$, respectively, indicating that this is likely to

Figure 5.9. HPLC/MS/MS analysis of tamoxifen digest in gastric fluid without pepsin and MS/MS spectra of the four peaks corresponding to m/z 404.



be 3,4–dihydroxytamoxifen. The identification of this peak as 3,4–dihydroxytamoxifen was further confirmed by the results obtained using hybrid quadrupole time-of-flight mass spectrometry, which will be described later. Total ion chromatograms and extracted ion chromatograms were carefully examined. The major digest products were (E)–tamoxifen (m/z 372), 3,4–dihydroxytamoxifen (m/z 404), and the other products (m/z 404 and 360) described above. No other metabolites listed in Table 1.3 were detected.

5.2.3.4. Comparison of Gastric Fluid Products to Urine Samples from Breast Cancer Patients on Tamoxifen Treatment

The HPLC/MS method described above was used to analyze two extracts of urine samples from breast cancer patients. Both of these patients had been on tamoxifen treatment for more than two years. These urine samples were extracted the same way as described in Section 3.2.3. Total ion chromatograms were obtained. The accurate masses of five metabolites are shown in Table 5.1.

Mass ions of these metabolites listed in Table 5.1 were extracted from the total ion chromatograms. Retention times and intensities of these peaks from two patient urine samples are summarized in Table 5.2. As shown, tamoxifen was detected in both urine samples, and identified by matching the retention time with that of the standard in addition to comparing the electrospray mass spectra. Other metabolites were also detected and some are listed in Table 5.2. Since no standards for these metabolites were available at the time this experiment was done, tentative identification of these metabolites was based on their mass spectra. None of these peaks were detected in a blank urine sample from a healthy subject who was not undergoing tamoxifen treatment.

(E)–tamoxifen, a major product in the gastric fluid digestion, was not detected in these two patient samples. Low concentrations of 3,4–dihydroxytamoxifen (m/z 404) were detected at a retention time of 22.0 min in both urine samples, as well as the tamoxifen digest, as shown in Figure 5.10. This peak was further analyzed using hybrid

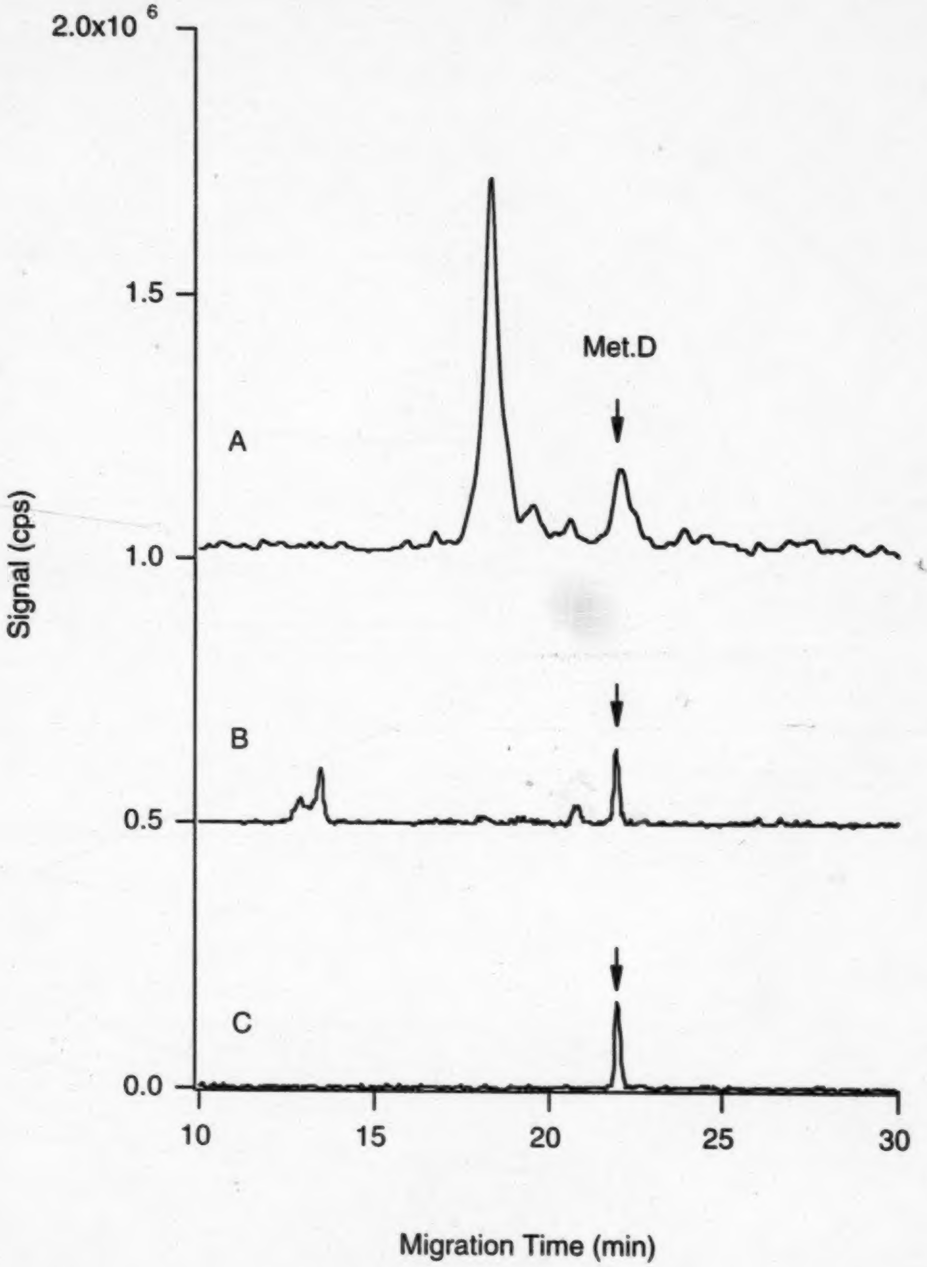
Table 5.1. The accurate masses of tamoxifen and some metabolites.

Compound	**Accurate Mass (g mol^{-1})**
N–desmethyltamoxifen	358.1968
tamoxifen	372.2238
4–hydroxytamoxifen	388.2232
tamoxifen–N–oxide	388.2232
3,4–dihydroxytamoxifen	404.2226

Table 5.2. Retention times and peak intensity of tamoxifen and metabolites detected in two urine samples.

Compound	Patient 1		Patient 2	
	Retention Time (min)	**Peak Height (cps)**	**Retention Time (min)**	**Peak Height (cps)**
N–desmethyltamoxifen	33.71	3.29×10^5	33.59	3.65×10^5
tamoxifen	34.91	5.31×10^5	34.73	2.02×10^6
tamoxifen-N-oxide	20.73	6.55×10^5	20.76	1.77×10^6
3,4-dihydroxytamoxifen	21.96	1.70×10^5	21.96	1.91×10^5

Figure 5.10. Extracted ion chromatograms of m/z 404 from (A) tamoxifen digest in gastric fluid without pepsin, (B) Patient 1 urine extract and (C) Patient 2 urine extract.



quadrupole-time-of-flight MS. One of the major advantages of hybrid quadrupole-time-of-flight MS is that accurate mass measurements can be made. The measured mass for these peaks was 404.2178, only a $1.2 \times 10^{-3}\%$ deviation from the theoretical mass, 404.2226, of 3,4–dihydroxytamoxifen. This confirms the identification of 3,4–dihydroxytamoxifen in the gastric fluid digest described above.

5.2.4 Conclusions

The transformation of tamoxifen at 37 °C in synthetic gastric fluid analyzed by high performance liquid chromatography with triple quadrupole mass spectrometry has been examined. The major transformation products detected were the E isomer of tamoxifen, 3,4–dihydroxytamoxifen, and several unidentified components having m/z 404. Addition of pepsin to the gastric fluid inhibited formation of all of these products. Two urine samples from breast cancer patients undergoing tamoxifen treatment were analyzed. Compared to the products of the gastric fluid digest, small amounts of 3,4–dihydroxytamoxifen and no (E)–tamoxifen were detected in urine samples.

This experiment demonstrates that use of extracted ion chromatograms from HPLC/MS can provide sensitive detection for complex samples with minimal sample preparation. Tamoxifen is relatively stable and can be further stabilized by adding proteins in an acidic environment. These results suggest that nonenzymatic transformation of tamoxifen in the human stomach may not be significant to the overall metabolism of tamoxifen in humans.

5.3 References

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Chapter 6

Conclusions and Future Work

6.1 Thesis Summary

The main focus of this thesis has been to show that tamoxifen metabolic profiles can be monitored in urine and serum samples from breast cancer patients using nonaqueous capillary electrophoresis with electrospray mass spectrometry. It has been shown here that tamoxifen and its metabolites in urine and serum samples can indeed be accurately and precisely measured using nonaqueous CE/ESI/MS.

In Chapter 2, a method for the analysis of tamoxifen and its metabolites was developed using nonaqueous CE/ESI/MS. CE separation was optimized with respect to the solvent and electrolyte of the buffer, injection time and voltage, and the running voltage. Metabolites were identified in patient urine and serum samples. The method was validated with respect to within-day precision by repetitively analyzing the same sample on one day, and to between-day precision by comparing migration times between many patients analyzed on different days and analyzing the same patient's extract on different days. Calibration curves were constructed from the analysis of standards and absolute concentrations were determined in a few patients. LC/ESI/MS was used as a comparison of analytical methods. The stability of samples within one day and over a larger period and relative comparisons between patients was also considered.

In Chapter 3, tamoxifen and several of its metabolites were extracted from 47 urine samples of patients with all stages of breast cancer. Analyses of these metabolites were performed by nonaqueous CE/ESI/MS. The use of nonaqueous CE/ESI/MS was validated as a qualitative and quantitative technique for clinical analysis of tamoxifen and its metabolites in urine samples from breast cancer patients. Several interesting correlations between patients' clinical characteristics and urinary tamoxifen metabolite profiles were observed. Patients with stage IV breast cancer have macroscopic metastatic tumors. Stage IV patients in the population had substantially higher tamoxifen–N–oxide metabolites than those patients with earlier-stage disease who had no evident macroscopic metastases. This observation raises the possibility that the breast cancer itself might influence tamoxifen metabolism, either directly by intratumoral metabolism, or indirectly by means of paraneoplastic effects on renal or hepatic function. However, paraneoplastic renal and hepatic complications are only rarely reported with breast cancer.

In Chapter 4, tamoxifen and several of its metabolites have been extracted from 80 serum samples of patients with all stages of breast cancer. Analyses of these metabolites were performed by nonaqueous CE/ESI/MS. Through these analyses it has been demonstrated that tamoxifen metabolic profiles in serum can be measured rapidly, reliably, and reproducibly with nonaqueous CE/ESI/MS. Serum tamoxifen and N–desmethyltamoxifen levels were lower in those patients with advanced breast cancer, suggesting that patients with large tumor burdens have altered tamoxifen metabolism compared to patients with early stage disease. The most probable explanation for this observation is that the presence of clinically detectable breast cancer deposits influence tamoxifen metabolism. One possible mechanism for this is intratumoral metabolism of tamoxifen reducing the serum levels of tamoxifen and N–desmethyltamoxifen. This proposed mechanism is supported by the results for the urine sample analyses.

In Chapter 5, two in vitro tamoxifen experiments were discussed. In the first, tamoxifen and its acid hydrolysis products were separated and tentatively identified by nonaqueous capillary electrophoresis with thermo–optical absorbance (CE/TOAD) and electrospray–ionization mass spectrometry (CE/ESI/MS). This experiment demonstrated that nonaqueous CE can separate tamoxifen and some of its degradation products without the use of surfactants. Nonaqueous CE/ESI/MS gives both the migration time and a mass spectrum, which aids in the identification of the degradation products. Acid hydrolysis is a convenient method of generating tamoxifen degradation products.

In the second experiment in Chapter 5, the transformation of tamoxifen at 37 °C in synthetic gastric fluid was analyzed by high performance liquid chromatography with triple quadrupole mass spectrometry. The major transformation products detected were the E isomer of tamoxifen, 3,4–dihydroxytamoxifen, and several unidentified components having m/z 404. Addition of pepsin to the gastric fluid inhibited formation of all of these products. The nonenzymatic transformation of tamoxifen in synthetic gastric fluid and the effects of pepsin (a digestive enzyme) on this process were investigated.

6.2 Future Work

After serum samples from nine patients with stage IV cancer were analyzed, a Cox regression analysis was used to explore the association between metabolite levels and the duration of clinical benefit experienced by these patients. The time to disease progression from the initiation of tamoxifen treatment was determined for each of these stage IV cancer patients. Results were promising in that significant associations were realized with concentrations of tamoxifen (p=0.0266) and N–didesmethyltamoxifen (p=0.0153) compared to survival curves for these patients. The obvious difficulty with these results however, was the insufficient number of patients (nine), let alone the insufficient number that had experienced disease progression (four). Therefore, these results were not meaningful. More patients' serum samples were analyzed, and when the total number of stage IV patients increased to 17, the same analysis was performed, this time with insignificant results. Of the 17 stage IV patients, there were only five whose cancer had progressed, and thus results were still inconclusive since there was still an insufficient number of patients with disease progression. These patients will have their data set reanalyzed with an additional year follow-up, and again in five years. The same metabolic profile will be used, but clinical outcomes will have varied. There is no need to reanalyze new serum samples from the same patients later on since it has been shown that in serum, steady state concentrations of tamoxifen and its metabolites remain stable over many years of therapy (1). These analyses in the future are expected to yield sufficient numbers of events (progression of advanced disease, or recurrence of early stage disease) to explore relationships between tamoxifen metabolite profiles and clinical outcomes.

6.3 References

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